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The Effects of in Vitro Fish Oil and Dietary Fish Meal Supplementation on Microdomains Within Bovine Luteal Cells

Michele Renae Plewes

UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

THE EFFECTS OF IN VITRO FISH OIL AND DIETARY FISH MEAL SUPPLEMENTATION ON MICRODOMAINS WITHIN BOVINE LUTEAL CELLS

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Michele Renae Plewes

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Accepted by the Doctoral Committee

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Patrick D. Burns, Ph.D., Research Advisor

_____

Mark P. Thomas, Ph.D., Committee Member

_____

James M. Haughian, Ph.D., Committee Member

_____

Richard M. Hyslop, Ph.D., Faculty Representative

Date of Dissertation Defense _____

Accepted by the Graduate School

_____

Linda L. Black, Ed.D.
Associate Provost and Dean
Graduate School and International Admission

ABSTRACT

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Lipid microdomains are regions of plasma membrane rich in cholesterol and sphingolipids, ranging in size from 10–200 nm in diameter. These structures serve as platforms to facilitate co-localization of intracellular signaling proteins during agonist-induced signal transduction. The omega-3 polyunsaturated fatty acids are a distinct class of fatty acids that have been shown to incorporate into lipid microdomains, disrupting the structural integrity of domains, and possibly attenuation of cell signaling. Fish byproducts, such as fish oil or meal, are a rich source of omega-3 polyunsaturated fatty acids that can be supplemented into cell cultures and diets of breeding females as a means for incorporating fatty acids into reproductive tissues. In chapter 2, we report that 0.03% (v/v) fish oils disrupt the spatial distribution of lipid microdomain and increases the lateral mobility of the prostaglandin (PG) $F_{2\alpha}$ (FP) receptor, which was unaffected by $PGF_{2\alpha}$ treatment. In charter 3, we report that fish oil inhibited $PGF_{2\alpha}$-induced mitogen-activated protein kinase (MAPK) signaling and disrupt $PGF_{2\alpha}$-induced receptor internalization and endosomal trafficking of the FP receptor in bovine luteal cells. In chapter 4, we examined the effect of individual omega-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on disruption of lipid microdomains and lateral mobility of the FP receptor. We found that 10 and 100 μM

EPA and DHA equally disrupts lipid microdomains and increase the lateral mobility of the FP receptor. Additionally, this disruption was equivalent to media supplementation of fish oil. Taken together, we found that supplementing cultured bovine luteal cells with fish oil leads to disruption of lipid microdomains, localization, and increases lateral mobility of the FP receptor, while attenuating $PGF_{2\alpha}$-induced MAPK signaling and receptor internalization. The effect of dietary fish meal supplementation on structural integrity of lipid microdomains, lateral mobility of the FP receptor, and luteal sensitivity to intrauterine infusions of $PGF_{2\alpha}$ are reported in Chapters 5 through 7. In Chapter 5, We report that fish oil and meal disrupted both lipid-lipid and lipid-protein interactions within lipid microdomains, translocating ganglioside, GM_1, and critical structural proteins (flotillin and caveolin) from lipid microdomain regions of plasma membrane into bulk lipid fractions. Additionally, we show that omega-3 polyunsaturated fatty acids, EPA and DHA, incorporated into bulk lipid fractions forming EPA- and DHA-rich domains. These EPA- and DHA-rich domains have a poor affinity for cholesterol, which resulted in translocation of cholesterol from bulk lipid into lipid microdomains. Next, we examined the effect of dietary supplementation on spatial distribution of lipid microdomains and the lateral mobility of the FP receptor. In Chapter 6, it was demonstrated that dietary supplementation of fish meal effected the fatty acid composition in luteal cells which lead to disruption in spatial distribution of lipid microdomains, and increased the lateral mobility of the FP receptor. Lastly, the final experiment investigated the effects of supplementation of fish meal on the structural and functional regression of the corpus luteum following intrauterine infusion of low doses of $PGF_{2\alpha}$. In chapter 7, we report that dietary supplementation of fish meal decreases luteal

sensitivity to intrauterine infusions of $PGF_{2\alpha}$ that prevented 54% of CL from functional regression and maintained progesterone production (progesterone per CL volume) from 0 to 48 h. Taken together, manipulation of lipid microdomains, FP receptor dynamics, and decreased luteal sensitivity to $PGF_{2\alpha}$ may lead to the development of methods for increasing reproductive efficiency in breeding cattle.

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INTRODUCTION AND REVIEW OF THE LITERATURE

It has been estimated that 20-30% of bovine embryos die within the first 30 days of gestation (1). This loss of potential pregnancies contributes to the millions of dollars lost annually in the United States dairy and beef industries due to the decrease in milk and meat production. There are multiple factors that contribute to early embryonic mortality including failure of fertilization, chromosomal abnormalities, embryo viability, failure of placental attachment, and failure in maternal recognition of pregnancy (2). The mechanisms by which embryo mortality occurs are still largely unknown; however, a likely cause appears to be insufficient secretion of embryonic interferon τ to inhibit prostaglandin synthesis in the maternal endometrial tissue. As a result of inadequate suppression of maternal prostaglandin synthesis and secretion, the corpus luteum (CL) regresses resulting in the termination of the pregnancy.

The long-term goal of this study was to evaluate the use of omega-3 polyunsaturated fatty acids found in fish by-products as a method to disrupt the luteolytic actions of prostaglandin (PG) $F_{2\alpha}$ in cows and heifers around the time of breeding. The objectives were to examine the effects of fish oil and fish meal supplementation on 1) plasma membrane structure, 2) prostaglandin receptor mobility, and 3) luteal sensitivity to $PGF_{2\alpha}$. It was postulated that fish oil and fish meal supplementation affect the action of $PGF_{2\alpha}$ on CL function. This interference would lead to an increased window for

interferon τ production by the embryo, preventing the regression of the CL from $PGF_{2\alpha}$ signaling in luteal cells. Additionally, inclusion of fish by-products in the diet of breeding cattle may alter luteal sensitivity to $PGF_{2\alpha}$ during the time of maternal recognition of pregnancy, thereby increasing calving rates.

Outcomes from this study have significantly increased our knowledge of plasma membrane dynamics, $PGF_{2\alpha}$ receptor mobility, and luteal sensitivity to $PGF_{2\alpha}$ in bovine luteal tissue. Elucidating mechanisms to protect the CL from the luteolytic effects of prostaglandins in reproductive tissues lead to novel ways to increase bovine pregnancy rates, further saving the agriculture industry and consumers millions of dollars annually.

Estrous Cycle

The female bovine, cows and heifers, are a non-seasonal polyestrous species, continuously entering estrous every 18 to 24 days, unless interrupted by pregnancy, lactation, stress, or pathology. The estrous cycle is divided into four different phases: estrus (day 0), metestrus (days 1-5), diestrus (days 6-17), and proestrus (days 18-24; Figure 1).

During estrus, the bovine expresses a homosexual behavioral characteristic known as lordosis, in which the female will stand firmly braced for mounting by another cow or bull for mating. This phase of the estrous cycle last 18-20 hours.

Hormonally, within the estrous phase, estradiol and inhibin concentrations are high, and progesterone is absent. High concentrations of estradiol stimulate the release of gonadotropin releasing hormone (GnRH) surge from the hypothalamus. This release of GnRH triggers a surge of luteinizing hormone (LH) from the anterior pituitary gland which results in release of the ovum from the ovulatory follicle. Inhibin release from

follicle results in decline in follicle stimulating hormone (FSH). Ovulation occurs approximately 32 hours, following the onset of estrus.

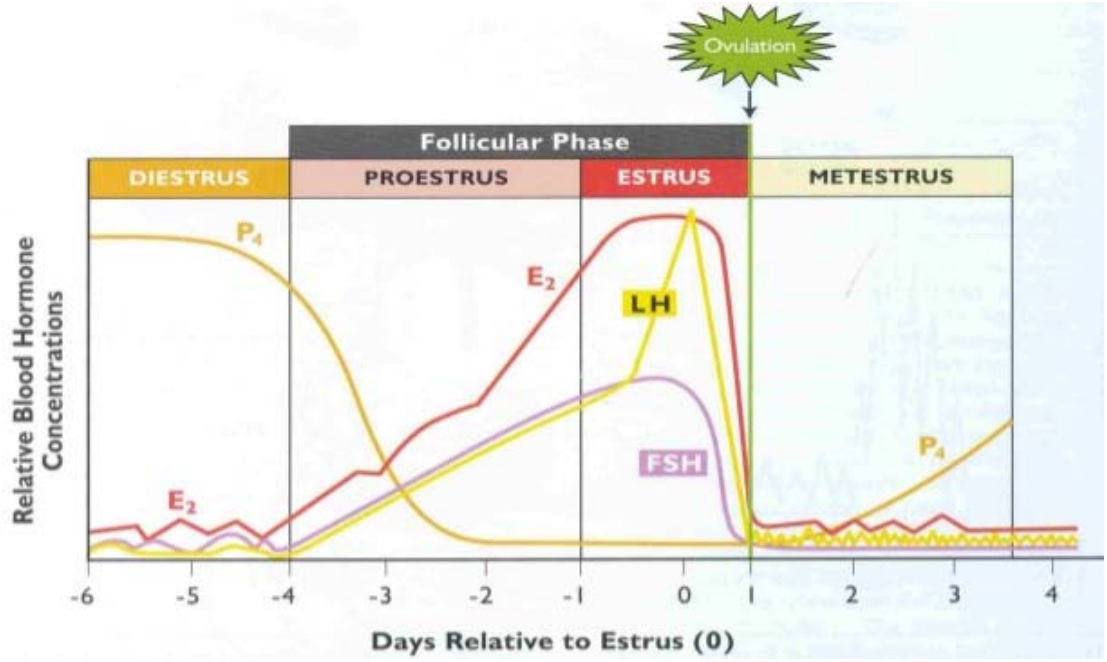

Figure 1. Schematic diagram of hormonal changes during the bovine estrous cycle. Image adapted from (3).

Following ovulation, the cycling female enters metestrus, which lasts 3-5 days. During this phase, the theca and granulosa cells from the ovulatory follicle differentiate in response to LH into the small and large luteal cells of the CL. Luteinization of theca and granulosa cells transforms steroidogenesis from estradiol-synthesizing cells into progesterone-biosynthesis. Hormonally, progesterone, which is synthesized from the CL, is rising, however, the CL is not considered mature and still undergoing angiogenesis. In addition to elevated progesterone, an FSH surge occurs which stimulates the first of three follicular waves.

Diestrus, is the longest phase of the bovine estrous cycle lasting from days 5-17 of the cycle. During this phase, there are two to four waves of follicular growth in response to elevated FSH concentrations, which varies among females. Within this follicular wave,

one follicle from a group of emerging follicles becomes the dominant follicles and causes the regression of the remaining follicles. Additionally, the dominant follicle secretes estradiol, however, luteal progesterone during diestrus prevents ovulation from proceeding. Additionally, the CL matures, and the steroidogenic cells of the CL continue to synthesize and secrete progesterone. In addition to secreting progesterone, the large luteal cells begin to express prostaglandin (PG) $F_{2\alpha}$ (FP) receptors for the luteolysin $PGF_{2\alpha}$. At the end of diestrus, if an embryonic signal is absent, a luteolytic cascade initiates both function (halt of progesterone biosynthesis) and structural (apoptosis) regression of the CL.

The last phase of the estrus cycle is referred to as proestrus. Within this phase, the CL is regressing, resulting in a decline in progesterone. Additionally, estradiol and inhibin are increasing as the new follicles begin to grow and develop steroidogenic capabilities. Moreover, decrease in progesterone results in pulsatile secretion of GnRH from the hypothalamus causing stimulation of LH and FSH release, preparing the female for ovulation and another estrous cycle.

The Corpus Luteum

The CL (Figure 2) is a transient endocrine gland formed from the ovulatory follicle following ovulation. The anterior pituitary secretes a surge of luteinizing hormone in response to hypothalamic GnRH, which is responsible for follicular rupture and the reprograming of follicular theca and granulosa cells into steroidogenic luteal cells. The morphological reshaping and migration of the granulosa and thecal cells is referred to as luteinization and serves as the final developmental stage for these cells. There are two morphologically distinct steroidogenic cells within the CL: the large and small luteal

cells. During angiogenesis of the CL, the ovarian granulosa cells differentiate into large luteal cells, and the theca cells differentiate into small luteal cells (4). Together, these cells account for approximately 70% of the volume of the CL, with the small luteal cells accounting for 28% and the large luteal cells accounting for 40% of total cell volume (5, 6).

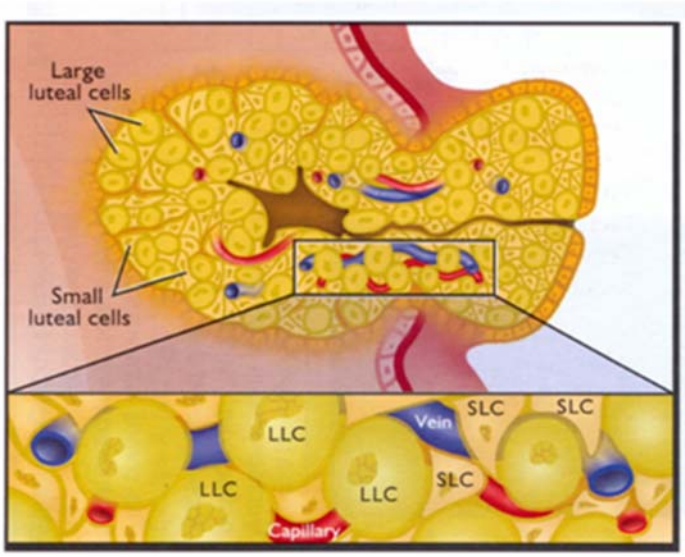

Figure 2. Model of the steroidogenic small and large luteal cells within a functional bovine corpus luteum. (CL; Figure modified from (7)). In the bovine CL, the small (SLC) and large luteal cells (LLC) are dispersed within the endothelial, fibroblasts, and immune cells (cell types not shown in figure) creating a mixed network of cells to create a functional CL.

The steroidogenic cells of the CL function to synthesize and secrete the steroid progesterone. Progesterone is a steroid hormone that is essential for the establishment and maintenance of pregnancy in the cow or heifer (8). Progesterone release from the CL blocks the estrogen-induced pituitary release of gonadotrophins and prepares the uterus for pregnancy (9). In the absence of an embryo, the endometrium releases a series of pulses of prostaglandin (PG) $F_{2\alpha}$ late in the estrous cycle (~ days 15 – 18), which causes regression of the CL. In the pregnant cow, the embryo must inhibit uterine $PGF_{2\alpha}$ secretion to maintain the structure and function of the CL. A schematic representation of

progesterone concentration throughout the estrous cycle in the non-pregnant and pregnant female is shown in Figure 3. Embryonic mortality often occurs when a viable embryo fails to effectively control $PGF_{2\alpha}$ secretion, which results in the regression of the CL and termination of the pregnancy (10). For that reason, manipulating luteal sensitivity to $PGF_{2\alpha}$ after mating may prevent early embryonic losses in cattle.

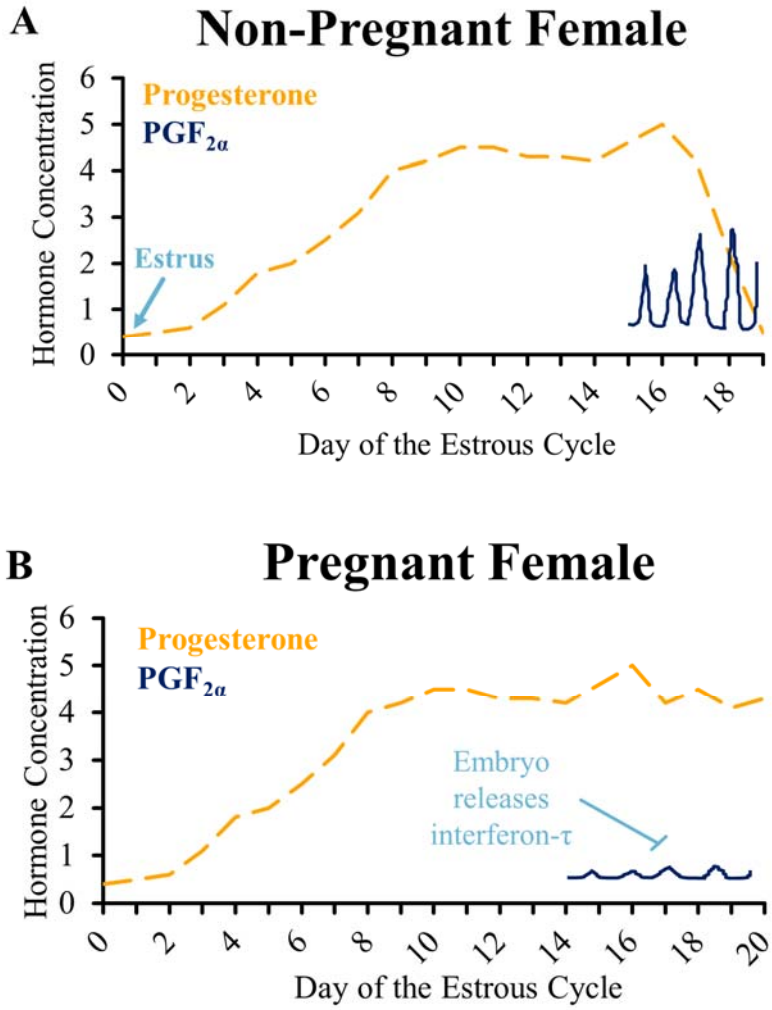

Figure 3. A schematic representation of progesterone concentration throughout the estrous cycle in the non-pregnant and pregnant female. Day 0 represents the onset of estrus and "behavioral heat". Approximately 32 h following onset of behavioral heat the female will ovulate and reprograming of follicular cells giving rise to the steroidogenic luteal cells. Dashed line (orange) represents serum progesterone concentration (ng/mL). The solid line (blue) represents uterine pulses prostaglandin (PG) $F_{2\alpha}$ late in the estrous cycle. Luteolytic pulses of $PGF_{2\alpha}$ leads to regression of the gland and decline in serum progesterone concentrations, allowing for another opportunity to become pregnant. In the pregnant cow, the embryo releases interferon τ which diminishes uterine $PGF_{2\alpha}$ secretion to maintain the structure and function of the CL.

Embryo viability plays a vital role in maternal recognition of pregnancy in the bovine. Early in pregnancy, the trophoblastic cells of the embryo must produce the cytokine interferon τ for successful implantation (1). This protein suppresses $PGF_{2\alpha}$

secretion, preventing the regression of the CL which is required for successful pregnancy (Fig 3). The embryo must produce appropriate concentrations of interferon τ by 17-19 days (show in Fig3) following estrus to prevent luteolysis (11, 12). Administration of interferon τ to breeding females has been shown to improve pregnancy rates in a few studies (13, 14). However, this approach requires daily administration of the protein which is impractical in large dairy or range beef cattle operations. Thus, altering the sensitivity of the CL to $PGF_{2\alpha}$ may be a logical approach to improve reproductive performance.

Regression of the CL is also referred to as luteolysis which has two distinct mechanisms: structural and functional regression. Functional regression refers to the decrease in progesterone biosynthesis, while structural regression refers to the degeneration of the gland (15). Prostaglandin $F_{2\alpha}$ is the endogenous luteolysin in domestic ruminants (16-19). It is secreted from the uterus in a series of pulses late in the estrous cycle, causing regression of the CL Fig 3 (20-22). In non-pregnant cows, $PGF_{2\alpha}$ binds to the prostaglandin $F_{2\alpha}$ (FP) receptor which is a seven-helix, G-protein coupled, membrane-bound receptor expressed on the plasma membrane of luteal cells (23-25). The binding of $PGF_{2\alpha}$ to its receptor initiates the phosphatidylinositol-phospholipase C intracellular signaling pathway that leads to the inhibition of progesterone synthesis and induction of apoptosis within the CL (26). A proposed model of $PGF_{2\alpha}$ signaling in the large luteal cells is shown in Figure 4. However, the interactions of ligand-bound receptors and associated heterotrimeric G-proteins that lead to activation of phospholipase C in bovine luteal cells are largely unknown.

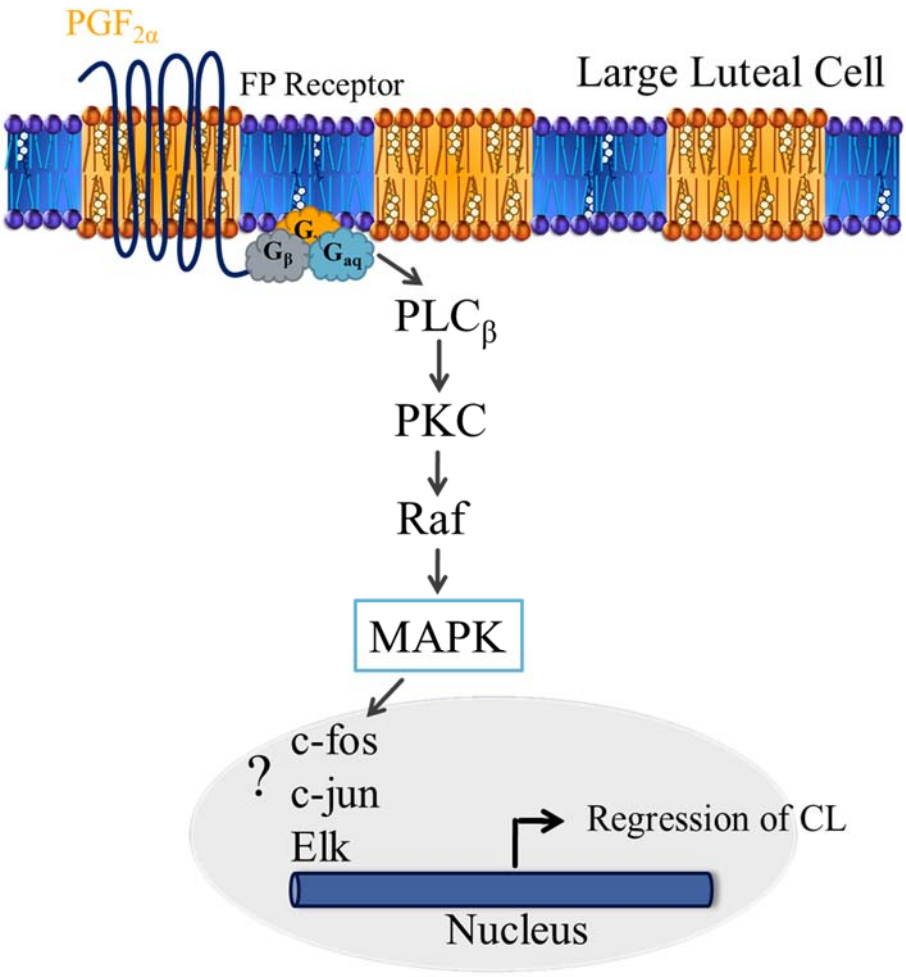

Figure 4. Proposed model of PGF_{2alpha} signaling in the large luteal cells. The bovine FP receptor is a transmembrane seven-helix G-protein-coupled receptor highly abundant on large luteal cells. The FP receptor interacts with heterotrimeric G-protein alpha subunit q, leading to activation of phosphatidylinositol-phospholipase C beta (PLCβ), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) signaling, and ultimately triggering apoptotic pathways, causing regression of the CL.

Progesterone Biosynthesis

Cholesterol is the precursor to all steroid hormones (27). Although steroidogenic luteal cells can synthesize cholesterol *de novo*, the majority of the cholesterol utilized for progesterone synthesis comes from the blood in the form of lipoproteins, Figure 5. Lipoproteins bind to membrane-bound receptors on the plasma membrane of luteal cells and are endocytosed (28). Cholesterol is sorted from lipoproteins within endosomes and

either trafficked to the mitochondrion, which is the site of the first enzymatic reaction, or stored as cholesterol esters in lipid droplets (27, 29). At the mitochondrion, cholesterol is transported across the outer and inner membrane by steroidogenic acute regulatory protein (STARD1), the rate-limiting step in steroidogenesis (30). Cholesterol side-chain cleavage enzyme (CYP11A1) is located on the inner mitochondrial membrane and catalyzes conversion of cholesterol to pregnenolone. Pregnenolone exits the mitochondrion and enters the endoplasmic reticulum, where it is converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3BHSD) (27). Progesterone is then secreted from the CL, and has many functions which include inhibiting the surge of GnRH secreted into the hypophyseal portal vasculature, blocking estradiol-induced pituitary release of gonadotrophins (9), regulating uterine function and promoting embryonic development during early pregnancy (31, 32).

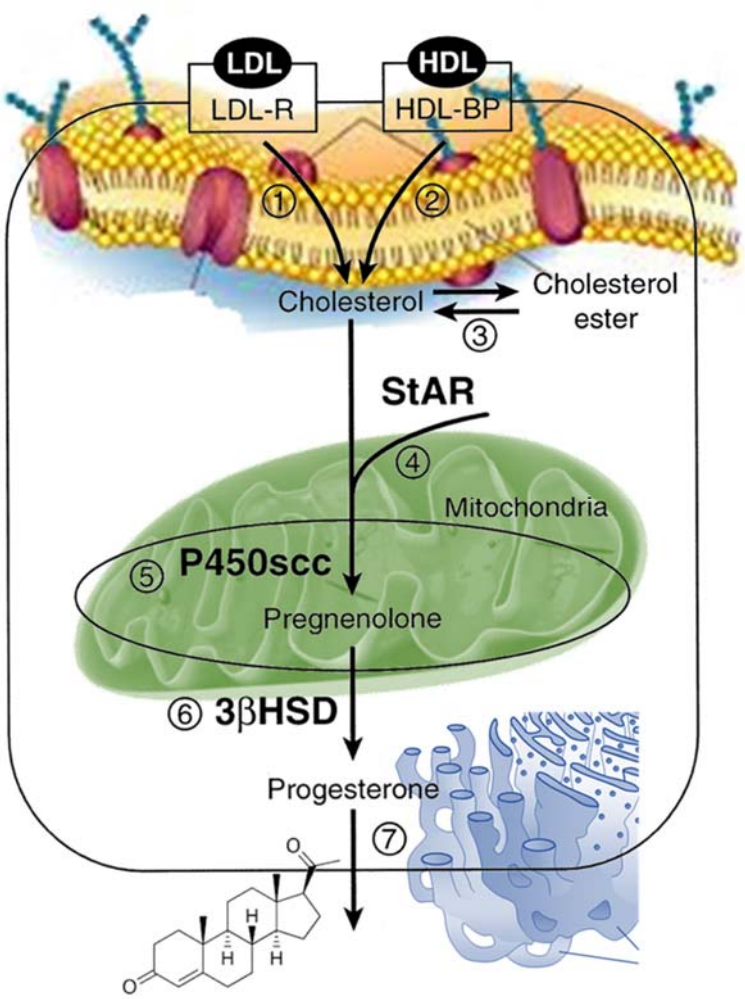

Figure 5. Schematic representation of progesterone biosynthesis in the large bovine luteal cell. Low-density Lipoprotein (LDL), High-density Lipoprotein (HDL), Steroidogenic acute regulatory protein (StAR), Cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD).

Omega-3 Polyunsaturated Fatty Acids

Nutrition, especially dietary energy intake (or energy balance), plays a critical role on reproductive performance of cattle (33-37). The omega-3 polyunsaturated fatty acids are a distinct class of long-chain fatty acids with a double bond at the third carbon from the methyl end of the acyl chain. There are three omega-3 fatty acids that have a significant impact on cellular physiology and possibly reproduction: α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA; Figure 6). Numerous

studies have shown beneficial effects of omega-3 polyunsaturated fatty acids on ovarian (38, 39) and uterine function in the bovine (40, 41). Additionally, supplementation of fats having a high percentage of omega-3 polyunsaturated fatty acids has been reported to suppress oxytocin-induced uterine synthesis of $PGF_{2\alpha}$ in cattle, which may contribute to a reduction in embryonic mortality (42, 43). The mechanism by which a diet high in omega-3 polyunsaturated fatty acids alter reproductive performance is still largely unknown.

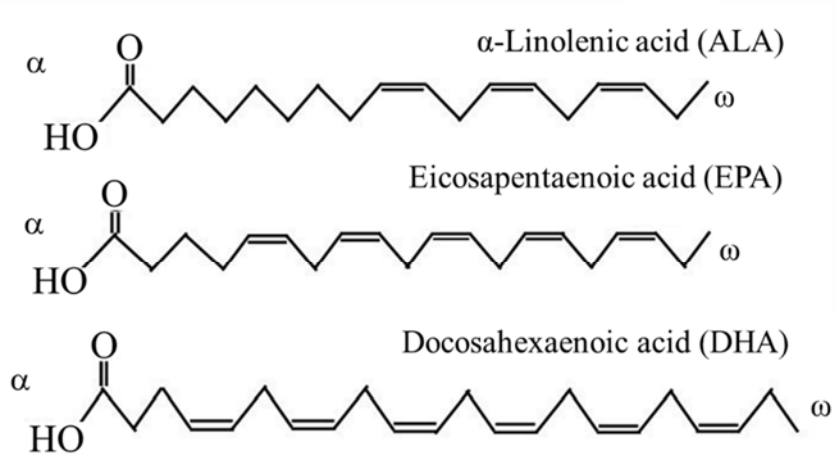

Figure 6. Omega-3 polyunsaturated fatty acids. Long chain fatty acids have a carboxyl end (alpha), which is the beginning of the chain, and a methyl end (omega), which is the terminal end. The naming of these fatty acids is indicated by the location of the first double bond on the methyl or carboxyl end. Polyunsaturated fatty acids are fatty acids that contain more than one double bond within the chain. The ω-3 fatty acids are polyunsaturated fatty acids comprised of a distinctive class of long-chain polyunsaturated fatty acids with a double bond at the third carbon atom from the methyl end. The three omega-3 polyunsaturated fatty acids fatty acids primarily involved in cellular physiology which are α-linolenic acid (ALA; 18:3), eicosapentaenoic acid (EPA; 20:5), and docosahexaenoic acid (DHA; 22:6). Omega-3 polyunsaturated fatty acids act as precursors of signaling molecules, which modulate microdomain composition within the plasma membrane, receptor signaling, and gene expression in bovine luteal cells.

The omega-3 class of polyunsaturated fatty acids are essential fatty acids involved in numerous biological processes such as inflammation (44, 45), cell signaling (46), and protection against oxidative stress (47). Long-chain polyunsaturated fatty acids such as

omega-3 fatty acids can be incorporated into glycerophospholipids and increase membrane fluidity (48-50). Changes in membrane order or fluidity has been reported to affect ligand affinity and subsequent ion flux for the acetylcholine receptor (51). In addition to altering membrane fluidity, these fatty acids have been reported to disrupt lipid microdomain composition, affect mobility of membrane-bound receptors, and decrease cell signaling (52-54). Fish oil and meal is a rich source of omega-3 polyunsaturated fatty acids, specifically EPA and DHA. We and others postulate that long-chain polyunsaturated fatty acids from fish by-products may incorporate into the membranes of bovine luteal cells, disrupting membrane dynamics, lateral mobility of the FP receptor, and ultimately decreasing luteal sensitivity to $PGF_{2\alpha}$.

Plasma Membrane and Lipid Microdomains

The plasma membrane of cells is composed of a lipid bilayer containing cholesterol, sphingolipids and glycerophospholipids (55-57). The lipids of the bilayer are not homogenous but rather segregated into microdomains (58, 59). A schematic representation of lipid microdomains is shown in Figure 7. Lipid microdomains are regions on the plasma membrane rich in cholesterol and sphingolipids, ranging in size from 10 to 200 nm in diameter (60, 61). There are two major types of lipid microdomains that have been described for biological membranes (62, 63). Linear domains, often referred to as membrane rafts or lipid rafts, are discrete cholesterol and sphingolipids-rich regions associated with the outer leaflet of the membrane. Curvilinear domains, also known as caveolae, appear to be associated with both outer and inner leaflets of membrane that are enriched with cholesterol and the protein caveolin at a 1:1 molar ratio. Both of these domains are involved in cellular trafficking, endocytosis/exocytosis,

maintenance of cholesterol homeostasis, and serve as signaling platforms during signal transduction (64-66). Additionally, both linear and curvilinear microdomains play a pivotal role in regulating G-protein-coupled receptor signaling in mammalian cells (67).

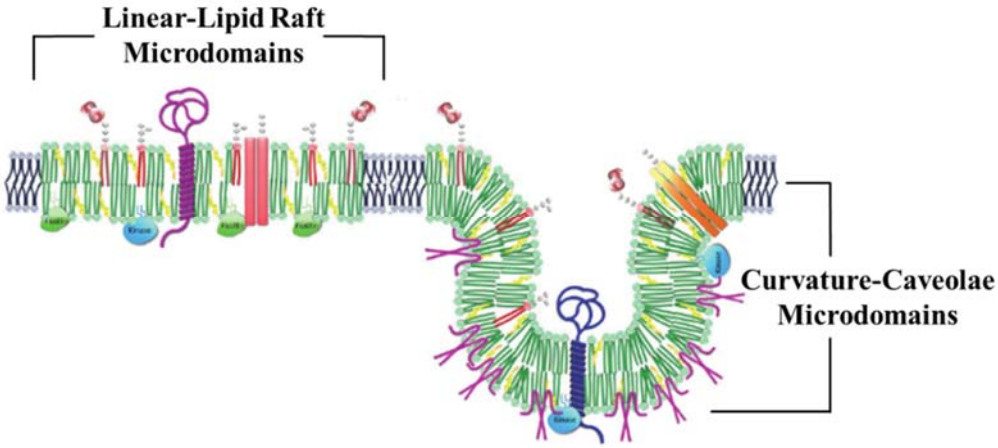

Figure 7. A schematic representation of linear and curvature lipid microdomains. Representation of the different lipid microdomains (green) and bulk-lipid membrane (grey). Lipid microdomains are highly ordered domains composed of cholesterol (yellow) and sphingolipids (green). Flotillin-1/2 (green circle) is the structural protein abundant in linear domains, and caveolin-1 (purple hairpin-like structure) is the structural protein responsible for the curvature of caveolae domains.

Membrane bound receptors, including G-protein-coupled receptors, are associated with lipid microdomains in both ligand-bound and unbound states (67). Moreover, heterotrimeric G-protein alpha subunits reside in lipid microdomains as reviewed in depth by Chini *et al,* (68). The cellular functions of these domains are still being resolved but may allow for the co-localization of receptors with their associated heterotrimeric G-protein, leading to the activation of downstream signaling. Thus, disruption of lipid-lipid, lipid-protein, or protein-protein interactions may alter downstream signaling. Figure 8 shows a model depicting omega-3 polyunsaturated fatty acid-induced disruption of lipid-lipid, lipid-protein, or protein-protein interactions in bovine luteal cell membranes.

Numerous studies have demonstrated that dietary supplementation with fish by-products increases plasma concentrations and thus the percentage of omega-3 fatty acids

incorporated into the plasma membrane of cells (53, 69-73) that remodel lipid microdomains (74). Additionally, fish oil supplementation alters membrane fluidity (71, 73) which influences lateral mobility of membrane-bound receptors. Taken together, incorporation of omega-3 fatty acids from fish oil or meal could affect the composition of lipid microdomains of bovine luteal cells which may lead to the alteration of lateral mobility of membrane-bound receptors and reduce $PGF_{2\alpha}$ in activation of the FP receptor activation.

The mechanism by which fish oil or meal affects lipid microdomain structure is still largely unknown. It is postulated that long chain polyunsaturated fatty acids, specifically the omega-3 fatty acids EPA and DHA, may play a key role in the disruption of the structural integrity of lipid microdomains. Generally, long-chain fatty acids associated with microdomains are saturated, allowing for tight packing, thereby increasing lipid order (75). The long chain omega-3 polyunsaturated fatty acids prevalent in fish oil can incorporate into the lipid microdomains (53, 74), which may increase the fluidity of this ordered region of the plasma membrane.

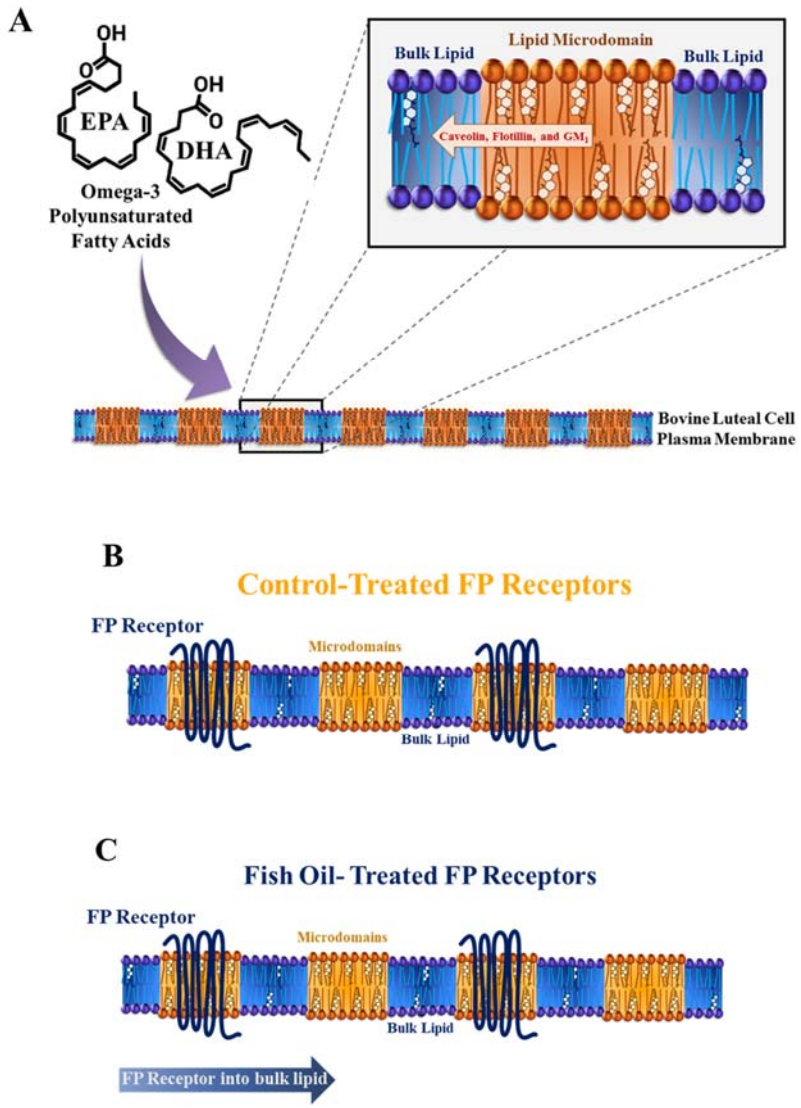

Figure 8. Incorporation of omega-3 polyunsaturated fatty acids into plasma membrane of bovine luteal cells. Panel A: Incorporation of EPA- and DHA-containing glycerophospholipids into biological membranes displace cholesterol from bulk lipid regions into lipid microdomains. The poor affinity for these long-chain polyunsaturated fatty acids with cholesterol creates EPA- and DHA- glycerophospholipid domains, or patches, leading to delocalization of microdomains markers (GM_1, caveolin-1 and flotillin-1/2) the bulk lipid membranes. Panel B: Proposed model for localization of the FP receptors in bovine luteal cells. The FP receptor is thought to reside in lipid microdomains. Panel C: Proposed model for localization of fish oil-treated FP receptors in bovine luteal cells. It is postulated that incorporation of omega-3 polyunsaturated fatty acids into the plasma membrane of luteal cells results in a disruption of lipid microdomains and displacement of the FP receptor into bulk lipid membranes.

Alteration of membrane fluidity or microdomain structure with fish oil or meal may affect mobility of integral and peripheral proteins associated with the plasma membrane. Likewise, disruption of lipid microdomains, specifically caveolae domains, may prevent caveolae-induced receptor internalization. Additionally, another mechanism may involve disruption of downstream signaling, preventing phosphorylation of the receptor for clathrin-mediated endocytosis.

Receptor Internalization

Proteins that interact with the lipid bilayer are categorized into two major groups: peripheral and integral. Integral proteins may span the bilayer, where they are referred to as transmembrane proteins. Transmembrane receptors span the plasma membrane, converting extracellular messages into intracellular responses. The G-protein-coupled receptors (GPCR) are the largest class of integral, membrane-bound receptors, interacting with a diverse group of agonists. Following agonist-mediated activation, GPCRs are internalized into the cell and sorted in endosomes for degradation (76, 77) or recycling to the plasma membrane (78). Receptor internalization functions as a key mechanism for signal desensitization (79), but has also been reported to resensitize a cell's GPCRs to ligand by recycling receptors back to the membrane (80). The mechanism that regulates agonist-induced internalization has been well characterized for GPCRs (68, 81-85). However, the mechanism that regulates both FP receptor internalization and sorting in reproductive cells is still unknown. A proposed model for FP receptor agonist-induced internalization and endosomal trafficking is shown in Figure 9.

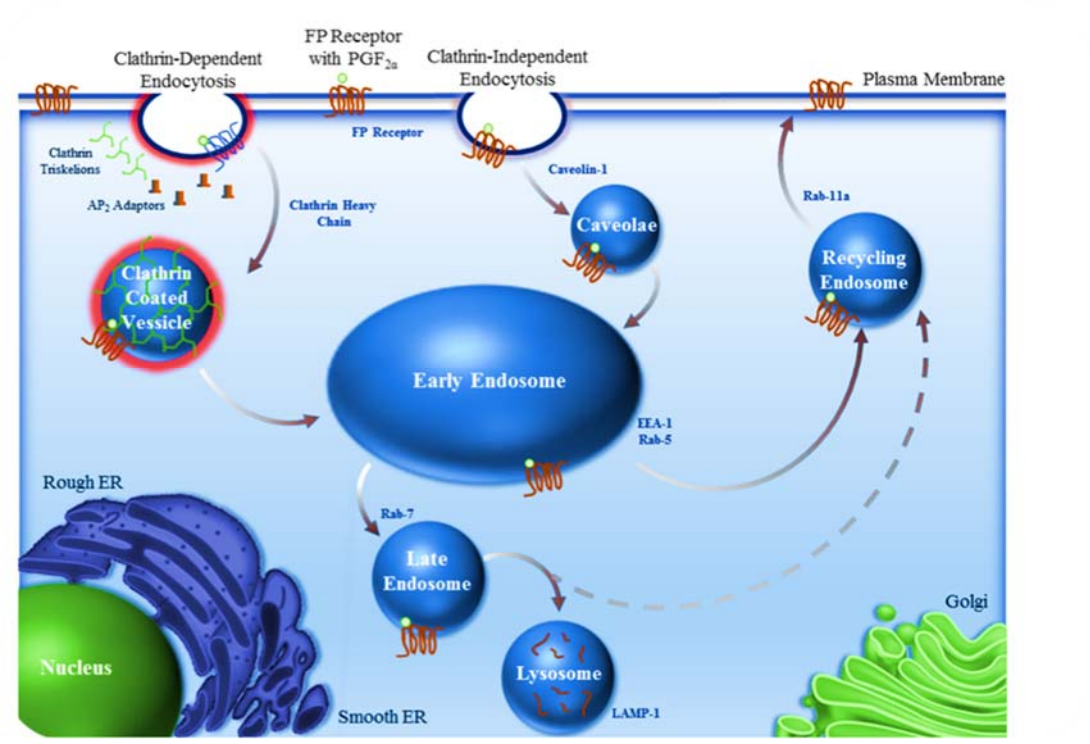

Figure 9. Proposed model for prostaglandin F_{2alpha} receptor agonist-induced internalization and endosomal trafficking. G-protein-coupled receptors utilize clathrin-dependent and/or clathrin-independent mechanisms for agonist-induced receptor internalization. Following internalization, the receptor is further trafficking to early endosomes and sorting, where its fate is degradation; late endosome trafficking, or resensitization/recycling to the membrane; recycling endosomes.

G-protein-coupled receptors have been shown to utilize both clathrin-dependent and clathrin-independent, agonist-mediated, receptor internalization. Clathrin-dependent internalization normally occurs at specialized sites of the plasma membrane, where clathrin-triskelion-coated vesicles are assembled, internalizing membrane-bound receptors for sorting. Caveolae-raft-mediated endocytosis is a clathrin-independent mechanism that uses lipid microdomains to internalize membrane receptors, including GPCRs, and cargo into the cell. Omega-3 polyunsaturated fatty acids have been reported to affect the cellular cytoskeleton (86), possibly influencing the lateral mobility of clathrin-coated pits through the cortical actin network. Here I postulated that omega-3

polyunsaturated fatty acids disrupt lipid microdomains, potentially influencing signaling and receptor internalization.

Specific Aims

The corpus luteum is a transient endocrine gland that synthesizes and secretes progesterone, a hormone essential for the establishment and maintenance of pregnancy. An estimated 20 to 30% of embryos die within the first 30 days of gestation, causing a significant loss in meat and milk production. The omega-3 polyunsaturated fatty acids are a distinct class of fatty acids that have been shown to employ beneficial effects on reproductive performance. However, the mechanism by which omega-3 polyunsaturated fatty acids exert beneficial effects is unknown. Fish by-products are a rich source of omega-3 polyunsaturated fatty acids, which have been shown to influence on cellular physiology as described above. In addition, omega-3 polyunsaturated fatty acids incorporate into biological membranes, which may lead to alterations in structure and membrane fluidity. Understanding the mechanism by which omega-3 polyunsaturated fatty acids exert beneficial effects on reproductive tissue, specifically the corpus luteum, is warranted to determine appropriate dietary recommendations for improving reproductive efficiency in mammalian females.

The core hypothesis driving our experiments was that dietary omega-3 polyunsaturated fatty acids supplementation in cattle disrupts lipid microdomains in luteal cells, leading to increased lateral mobility of the FP receptor, therefore inhibiting FP receptor internalization, sensitivity, and subsequent luteolytic PGF signaling. To test the hypothesis, we established the following objectives which examined membrane structure and dynamics of the plasma membrane, as well as, protein interactions,

specifically with the $PGF_{2\alpha}$ receptor. In addition to determining the effects of fish by-products on luteal membrane dynamic, the effects of dietary supplementation of fish meal on luteal sensitivity by administering intrauterine infusions of $PGF_{2\alpha}$ and measuring both structural and functional regression of the gland were determined. It was hypothesized that incorporation of omega-3 polyunsaturated fatty acids into biological membranes will disrupt the order and structure of biological membranes, increase membrane fluidity, and ultimately alter the properties of the $PGF_{2\alpha}$ receptor in bovine luteal cells. Taken together, inclusion of fish by-products in the diets of breeding females may decrease the luteal sensitivity to $PGF_{2\alpha}$. Decreasing the luteal sensitivity to $PGF_{2\alpha}$ may extend the window for maternal recognition of pregnancy, increasing pregnancy rates in the bovine.

Objective I examined the effects of fish oil on the structural integrity of lipid microdomains and localization of the prostaglandin (PG) $F_{2\alpha}$ (FP) receptor on the plasma membrane of bovine luteal cells. Fish oil contains a high percentage of omega-3 polyunsaturated fatty acids which may incorporate into biological membranes, affecting membrane dynamics. Incorporation of omega-3 fatty acids alter plasma fatty acid composition, affecting membrane fluidity and microdomain structure. We hypothesized that disruption of lipid microdomains may affect the protein-lipid interactions of the FP receptor, increasing lateral mobility and decreasing $PGF_{2\alpha}$-induced receptor docking, cell signaling, and receptor internalization. Data on this objective can be found in Chapters 2, 3, and 5. Developing a non-invasive mechanism for disrupting $PGF_{2\alpha}$ signaling in large luteal cells may lead to an increased window for maternal recognition of pregnancy.

Objective II determined the influence of individual omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on bovine luteal cell

plasma membrane dynamics. Fish oil obtained from cold water fishes is abundant in both EPA and DHA and supplementing the diet with fish oil or fish meal has been reported to affect lipid microdomain structure. However, which individual omega-3 fatty acid in fish oil affects lipid microdomain structure is largely unknown. We hypothesized that supplementation of individual EPA or DHA made it possible to determine which omega-3 fatty acids leads to observed spatial distribution of lipid microdomains and increased lateral mobility of the FP receptor. Data on this objective can be found in Chapter 4. Understanding the influence that individual omega-3 fatty acids, EPA and DHA, have on membrane structure is critical for making the appropriate dietary recommendations for improving reproductive efficiency in mammalian females.

Objective III determined the effects of inclusion of fish meal in the diet of beef cows on the lateral mobility of the prostaglandin (PG) $F_{2\alpha}$ (FP) receptor and membrane structure in luteal cells. Dietary fish meal supplementation increases omega-3 fatty acid composition in bovine luteal tissue. Therefore, it was anticipated that dietary supplementation of fish meal will allow for incorporation of omega-3 polyunsaturated fatty acids into the biological membranes of bovine luteal tissue, allowing for disruption of lipid microdomains. Dietary supplementation of fish meal may lead to the disruption of the structural integrity of lipid microdomains, decreasing the spatial distribution and altering localization of critical structural proteins. This disruption of lipid microdomains may alter the localization and lateral mobility of FP receptors in bovine luteal cells. Data on this objective can be found in Chapters 5 and 6. Disruption of lipid microdomains through inclusion of fish meal in the diets of breeding females may lead to novel feeding strategies for increasing reproductive success.

Objective IV determined the effects of inclusion of fish meal in the diet of beef cows on luteal sensitivity following administration of low doses of $PGF_{2\alpha}$. Late in the estrous cycle, uterine $PGF_{2\alpha}$ binds to receptors on large luteal cells of the corpus luteum, triggering an intracellular signaling cascade that leads to functional and structural regression of the gland. We hypothesized dietary supplementation of fish meal may decrease the luteal sensitivity to $PGF_{2\alpha}$, allowing for rebound in serum progesterone and sustained luteal diameter. Additionally, these data are found in chapter 7. Inclusion of fish meal in the diets of breeding females may lead to novel feeding strategies for decreasing luteal sensitivity to $PGF_{2\alpha}$, increasing the window for maternal of recognition.

These studies established a mechanistic insight into the beneficial effects of fish by-products on bovine luteal cells, which may lead to novel, non-invasive, and cost-effective feeding strategies for improving reproductive success in the bovine.

CHAPTER 2

EFFECT OF FISH OIL ON LATERAL MOBILITY OF PROSTAGLANDIN F_{2ALPHA} (FP) RECEPTORS AND SPATIAL DISTRIBUTION OF LIPID MICRODOMAINS IN BOVINE LUTEAL CELL PLASMA MEMBRANE *IN VITRO*.

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Contribution of Authors and Co-Authors

Manuscript in Chapter II

Author: Michele R. Plewes

Contributions: Developed and implemented the study design. Generated and analyzed data. Wrote first draft of the manuscript.

Co-Author: Patrick. D. Burns

Contributions: Helped conceive the study topic. Provided guidance on study design. Provided feedback data interpretation and manuscript preparation.

Co-Author: Peter E. Graham

Contributions: Helped conceive the study topic. Provided guidance on study design.

Co-Author: Richard M. Hyslop

Contributions: Provided feedback data interpretation and manuscript preparation.

Co-Author: B. George Barisas

Contributions: Helped conceive the study topic. Provided software program for analysis. Provided feedback data interpretation and manuscript preparation.

Abstract

Lipid microdomains are ordered regions on the plasma membrane of cells, rich in cholesterol and sphingolipids, ranging in size from 10 to 200 nm in diameter. These lipid-ordered domains may serve as platforms to facilitate co-localization of intracellular signaling proteins during agonist-induced signal transduction. The objectives of this study were to examine the effects of fish oil on 1) the spatial distribution of lipid microdomains, 2) lateral mobility of prostaglandin (PG) $F_{2\alpha}$ (FP) receptors and 3) lateral mobility of FP receptors in the presence of $PGF_{2\alpha}$ on the plasma membrane of bovine luteal cells *in vitro*. Bovine ovaries were obtained from a local abattoir and corpora lutea were digested using collagenase. In Experiment 1, lipid microdomains were labeled using cholera toxin subunit B Alexa Fluor 555. Domains were detected as distinct patches on the plasma membrane of mixed luteal cells. Fish oil treatment decreased fluorescent intensity in a dose dependent manner ($P < 0.01$). In Experiment 2, single particle tracking was used to examine the effects of fish oil treatment on lateral mobility of FP receptors. Fish oil treatment increased micro-and macro-diffusion coefficients of FP receptors as compared to control cells ($P < 0.05$). In addition, compartment diameters of domains were larger and residence times were reduced for receptors in fish oil treated cells ($P < 0.05$). In Experiment 3, single particle tracking was used to determine the effects of $PGF_{2\alpha}$ on lateral mobility of FP receptors and influence of fish oil treatment. Lateral mobility of receptors was decreased within 5 min following addition of ligand for control cells ($P < 0.05$). However, lateral mobility of receptors was unaffected by addition of ligand for fish oil treated cells ($P > 0.10$). The data presented provide strong

evidence that fish oil causes a disruption in lipid microdomains and affects lateral mobility of FP receptors in the absence and presence of $PGF_{2\alpha}$.

Introduction

Prostaglandin (PG) $F_{2\alpha}$ is the endogenous luteolysin in domestic ruminants (16-19). It is secreted in a series of pulses late in the estrous cycle from the uterus, causing regression of the corpus luteum (CL) (20-22). In non-pregnant cows, $PGF_{2\alpha}$ binds to the prostaglandin (PG) $F_{2\alpha}$ (FP) receptor which is a seven-helix, G-protein coupled, membrane-bound receptor located on luteal cells (23-25). The binding of $PGF_{2\alpha}$ to its receptor initiates the phosphatidylinositol-phospholipase C intracellular signaling pathway that leads to the inhibition of progesterone synthesis and induction of apoptosis within the CL (26). However, the interactions of ligand-bound receptors and associated heterotrimeric G-proteins that lead to activation of phospholipase C in bovine luteal cells are largely unknown.

The plasma membrane of cells is composed of a lipid bilayer containing cholesterol, sphingolipids and glycerophospholipids (55-57). The lipids of the bilayer are not homogenous but rather segregated into microdomains (58, 59). Lipid microdomains are regions on the plasma membrane of cells rich in cholesterol and sphingolipids, ranging in size from 10 to 200 nm in diameter (60, 61). Membrane bound receptors, including G-protein coupled receptors, have been reported to be associated with lipid microdomains in both ligand-bound and unbound states (67). Moreover, heterotrimeric G-protein alpha subunits have been reported to reside in lipid microdomains as reviewed in depth by Chini *et al,* (68). The cellular functions of these domains are still being resolved but may allow for the co-localization of receptors with its associated

heterotrimeric G-protein, leading to the activation of downstream signaling. Thus, disruption of lipid-lipid, lipid-protein, or protein-protein structure may cause alteration in downstream signaling.

Inclusion of marine fish oil into the diet has been reported to alter lipid dynamics within the plasma membrane of many cell types. Studies have shown that fish oil supplementation alters membrane fluidity (71, 73) which may influence lateral mobility of membrane bound receptors. Other studies have shown that omega-3 fatty acids, predominantly eicosapentaenoic and docosahexaenoic acid, both present in triglycerides found in fish oil, incorporate into plasma membranes of T-cells which have an effect on the fatty acid composition and morphology of the lipid microdomains (53, 87). This suggests that the incorporation of omega-3 fatty acids from fish oil could also affect the composition of lipid microdomains of bovine luteal cells which may lead to the alteration of lateral mobility of membrane-bound receptors. The objectives of this study were to determine the effects of fish oil on 1) the spatial distribution of lipid microdomains, 2) lateral mobility of FP receptors, and 3) lateral mobility of FP receptors in the presence of $PGF_{2\alpha}$ on the plasma membrane in bovine luteal cells *in vitro*.

Methods

Tissue Collection, Cell Preparation, and Cell Culture

Bovine ovaries containing a CL were collected at a local abattoir and transported to the laboratory at the University of Northern Colorado in 1× sterile PBS. The ovaries were then immersed in 70% ethanol to destroy any microorganisms that may be present from time of collection.

Using sterile techniques under a laminar flow hood, the CL was removed from the ovary and placed into a 60 mm^2 Petri dish containing ice-cold Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS, pH 7.34). The CL was dissected free of connective tissue and cut into approximately 1 mm^3 fragments. Approximately 1 g of tissue was placed into T-25 culture flasks containing 5 mL dissociation medium (HBSS supplemented with 2000 units of collagenase type 1 and 0.1% BSA) and incubated in a water bath at 37 °C with agitation for 45 min. Following incubation, the supernatant was removed and transferred to a sterile 15-mL culture tube. Cells were then washed 3× with sterile PBS, re-suspended in 10 mL of culture medium (Ham's F12 supplemented with fetal bovine serum (5%), insulin (5 µg/mL), transferrin (5 µg/mL), sodium selenate (5 ng/mL), 100 unit per mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (pH 7.34)), and placed on ice. Fresh dissociation medium was added to the remaining undigested tissue and incubated with agitation for an additional 45 min. The remaining cells were collected, washed 2× with sterile PBS, and combined with the previous sample. After the final wash, cells were re-suspended in 10 mL of culture medium.

Viability of cells was determined using trypan blue and cell concentration was estimated using a hemocytometer. Only preparations with a cell population of greater than 85% viability were used for each experiment. Cell cultures were maintained at 37 °C in an atmosphere of 95% humidified air and 5% CO_2.

Fish Oil Preparation for In Vitro Culture

Fish oil used for all experiments was extracted from a mixture of fish (Anchovy, Sardine, and Mackerell). Lipids in fish oil were pre-bound to BSA prior to the addition to culture. In brief, fish oil was added to culture medium containing 33 mg/mL fatty acid

free BSA at the appropriate dose for each experiment as described by Mattos *et al*. (88) Control medium was prepared using the same conditions as fish oil treatment, without the addition of fish oil.

Experiment 1: Effects of Fish Oil on Spatial Distribution of Lipid Microdomains

Mixed luteal cell cultures were prepared from four CL and plated in 35-mm glass bottom culture dishes at 5×10^4 cells/dish (n = 15 dishes/CL). Cells were incubated overnight at 37 °C in an atmosphere of 95% humidified air and 5% CO_2 to allow adhesion to glass cover slips. Culture medium was removed, and cells were treated with 0, 0.0003, 0.003, 0.03 or 0.3% (vol/vol) fish oil (n = 3 dishes/treatment) for 72 h to allow incorporation of fatty acids into membranes. Additional dishes (n = 6 dishes/CL) were incubated in 0% (vol/vol) fish oil to serve as positive and negative controls.

Lipid microdomain labeling and visualization. Lipid microdomains were labelled using a commercially available kit per manufacture's protocol (Vybrant Lipid Raft Labelling kit; Invitrogen, Carlsbad, CA, USA). In brief, cells were washed 3× with 1 mL PBS. After final wash, 1 mL ice cold culture medium was added to each dish containing 1 μg cholera toxin subunit B Alexa Fluor 555 conjugate. Dishes were incubated at 4 °C in the dark for 10 min. Cells were then washed 3× with PBS and 1 mL ice-cold culture medium containing anti-cholera toxin subunit B antibody (1:200 dilution) was added. Dishes were incubated at 4 °C for 15 min in the dark and subsequently washed 3× with PBS. After the final wash, 1 mL PBS was added to dishes and cells were immediately visualized. As a positive control, three dishes were treated with 10 mM beta-methylcyclodextrin (β-MCD) for 1 h under control conditions to deplete membrane

cholesterol and disruption of lipid microdomains. As a negative control, three dishes were treated with only anti-cholera toxin subunit B antibody.

An Olympus IX81 motorized inverted microscope (Tokyo, Japan), equipped with a 60× oil objective and 0.8× digital zoom was used to visualize lipid microdomains. Approximately 10 cells were randomly selected from each dish and 1 μm slice z-stacked images were generated from bottom to top of each cell. A 3-dimensional image of each cell was created using Fluoview version 4.3 software. Images were converted to 8-bit/channel TIFF format and were processed utilizing ImageJ (National Institutes of Health, Stapleton, NY, USA) analysis software. Mean cell fluorescence intensity was determined by outlining the cell boundary and subtracting background fluorescence.

Experiment 2: Effects of Fish Oil on Lateral Mobility of FP Receptors on Bovine Luteal Cells

Mixed luteal cell cultures were prepared from nine CL and cultured in 35-mm round bottom glass dishes at 5×10^4 cells/dish. Cells were cultured in control medium or medium supplemented with 0.03% (vol/vol) fish oil for 72 h. Following 72 h incubation at 37 °C, cells were then prepared for single particle tracking.

Biotin was added to FP receptor polyclonal antibody (101802; Cayman Chemical Company, Ann Arbor, MI, USA) using a commercially available kit per manufacture's protocol (DSB-X Biotin Protein Labeling Kit; Cayman Chemical Company). In brief, stock antibody solution was diluted to 0.5 mg/mL and desalted using a spin column prior to labeling. Two hundred microliters of desalted antibody was combined with 20 μL of freshly prepared 1 M $NaHCO_3$ and placed in a reaction tube. DSB-X biotin succinimidyl ester was reconstituted in 40 μL of DMSO, and 2 μL of the DSB-X biotin solution was

added to 200 μL of FP receptor polyclonal antibody. The derivatization reaction was carried out at room temperature for 1.5 h with constant stirring. Biotinylated antibody was collected using a spin column containing purification resin and centrifuged 5 mins at 1100 × g to remove unbound DSB-X. Labeling of antibody was verified using SDS-PAGE and western blotting (data not shown).

Biotinylated FP receptor antibody (1 μg) was added to each 35-mm dish and incubated at 37 °C in an atmosphere of humidified air and 5% CO_2 for 15 min. Medium was then decanted and 2 mL of 1× PBS was added to each dish, gently agitated for approximately 5 s, and decanted. This procedure was repeated 2× to remove unbound antibody. Cells were then incubated with 0.5 nM 605 quantum dots conjugated with streptavidin (Q10101MP; Invitrogen) at room temperature for 2 min. Cells were rinsed 6× using gentle agitation at 20 s intervals in 1 × PBS to remove unbound quantum dots.

A Zeiss confocal microscope equipped with a high-speed camera (Hamamatsu Photonics, Shizuoka, Japan) was used to record receptor mobility. Quantum dots were excited with UV light and the 605-nm emission was collected using the appropriate filter set. Receptors were observed with a 100× oil objective (N.A. = 1.4) and acquisition was set as an image size of 512 × 512 pixels (0.07 μm/pixel × 0.07 μm/pixel) with 1 × 1 binning. Receptors were recorded at 30 frames/s for a total of 870 frames per video.

Trajectories of individual receptors were generated using Video Spot Tracker v08.01 (University of North Carolina-Chapel Hill, Chapel Hill, NC, USA). Only trajectories with greater than 10 s were used for further analysis. These trajectories were given as X-Y pixel coordinates which were in measurements of 0.07 μm × 0.07 μm. Mean square displacement of trajectories was calculated and plotted as a function of time

to determine both micro-and macro-diffusion coefficients according to the equations reported by Daumas *et al*. (89).

Residence time of individual receptors was calculated as previously described by Barisas *et al*. (90). In brief, a sliding window analysis was used to determine the normalized variance in the position of the receptor within time windows. Windows were then translated alongside of particles time trajectory. The time of the inter-domain jumps was indicated by peaks and collected as n+1. The average diameter of an individual domain was calculated as previously described (89-91). Domain size was determined by taking the square root of the macro-diffusion coefficient × 4 residence time.

Experiment 3: Effects of Fish Oil and Prostaglandin $F_{2\alpha}$ on Lateral Mobility of FP Receptors on Bovine Luteal Cells

Mixed luteal cell cultures were prepared from seven CL and cultured in 35 mm round bottom glass dishes at 5×10^4 cells/dish. Cells were cultured in control culture medium or medium supplemented with 0.03% (vol/vol) fish oil for 72 h. Cells were then prepared for single particle tracking as described in Experiment 2.

Five luteal cells containing a minimum of 20 labeled FP receptors from each dish were randomly selected for recording. Following the identification of labeled cells, XYZ coordinates were saved to ensure consistent directional positioning along the cell membrane throughout the experiment. Selected cells were recorded without ligand at 0 min to determine initial diffusion coefficients, residence time, and domain size. Cells were then treated with 10-nM $PGF_{2\alpha}$ analog (cloprostenol sodium, Merck Animal Health, Madison, NJ, USA) and recorded at 5, 15, and 30 min post-treatment. Receptors were recorded for 29 s (30 frames/s) for a total of 870 frames per time point and analyzed as

described in Experiment 2. All visibly labelled FP receptors were counted at 0 and 30 min post- $PGF_{2\alpha}$ treatment to estimate percent decrease in receptor number present on the plasma membrane.

Statistical Analysis

Data sets were examined for normality using the Shapiro-Wilk test and transformed accordingly using natural logarithms prior to statistical analysis. All data are reported as least square means ± standard error of the mean and significance was declared at $P < 0.05$. In Experiment 1, effects of fish oil on fluorescent intensity of lipid microdomains were analyzed using one-way analysis of variance. The statistical model included CL, concentration of fish oil, cell and residual error. Corpus luteum was used as random variable in the model. The effects of fish oil concentration on fluorescent intensity were further characterized by using linear regression analysis. In Experiment 2, lateral mobility of FP receptors (micro- and macro-diffusion coefficients), domain sizes, and residence time of receptors were analyzed using one-way analysis of variance. The model included CL, cell, receptor and residual error as sources of variation. Corpus luteum was considered a random variable in the model. In Experiment 3, the effects of fish oil and $PGF_{2\alpha}$ on lateral mobility of FP receptors, domain sizes, and residence time were analyzed using one-way analysis variance. The model included CL, cell, receptor, time and residual error as sources of variation. Corpus luteum was considered as a random variable in the model. The effects of ligand on the disappearance of FP receptor on the plasma membrane 30 min post-treatment in Experiment 3 was analyzed using a one-way analysis of variance. The statistical model included CL, treatment (control, 0.03% fish oil, β-MCD), cell and residual error. Corpus luteum was used as random

variable in the model. Calculations were made using the mixed-model procedure or regression procedure of SAS. If main effects or interactions were significant, then means were separated using pre-planned *t*-tests and PDIFF option of SAS.

Results

Experiment 1: Effects of Fish Oil on Spatial Distribution of Lipid Microdomains on Bovine Luteal Cells.

Representative micrographs acquired from control, fish oil, and β-MCD treated luteal cells are shown in Figure 10A. Lipid microdomains were detected as distinct patches on the plasma membrane of bovine luteal cells. Fish oil and β-MCD treatment had a major impact on structural integrity of these domains. Mean cell fluorescent intensities of microdomains were greater in control cells as compared to cells treated with fish oil or β-MCD ($P < 0.05$). Fish oil decreased mean cell fluorescent intensity of microdomains in a dose-dependent manner ($P < 0.05$; Fig 10B). Increasing fish oil concentrations from 0.0003 to 0.3 % (vol/vol) resulted in a linear disruption of lipid microdomains. Furthermore, removal of membrane cholesterol using β-MCD resulted in further disruption within microdomains, decreasing fluorescent intensity, when compared to control treated cells ($P < 0.05$).

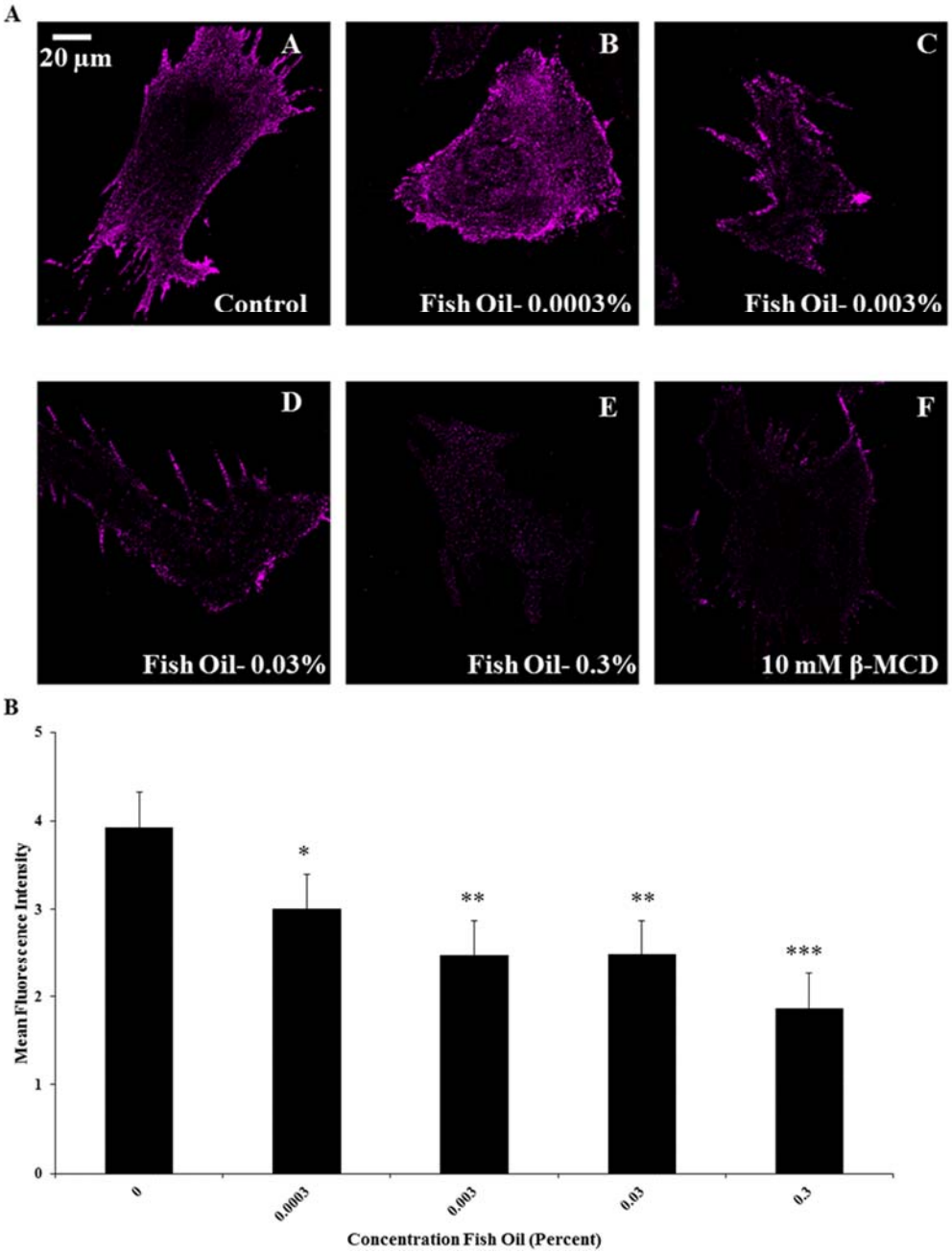

Figure 10. Effects of fish oil on spatial distribution of lipid microdomains. Panel A shows representative micrographs obtained from (A) control cells, (B) 0.0003% fish oil (vol/vol), (C) 0.003% fish oil (vol/vol), (D) 0.03% fish oil (vol/vol), (E) 0.3% fish oil (vol/vol), and (F) 10 mM beta-methylcyclodextrin (β-MCD). Panel B shows means of the fluorescent intensity for luteal cells obtained from control cells (n=49), 0.0003% fish oil (vol/vol; n=48), 0.003% fish oil (vol/vol; n=48), 0.03% fish oil (vol/vol; n=49), and 0.3% fish oil (vol/vol; n=47); control vs. fish oil; *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

Experiment 2: Effects of Fish Oil on Lateral Mobility of Prostaglandin $F_{2\alpha}$ Receptors on Bovine Luteal Cells.

Labeled FP receptors on the plasma membrane of a bovine luteal cell using biotinylated FP receptor antibody and 605 nm streptavidin quantum dots are shown in Figure 11. A representative trajectory from a control and fish oil treated cell is shown in Figure 12. Fish oil treatment had a significant influence on lateral mobility of FP receptors. Both micro- and macro-diffusion were increased in fish oil treated cells compared to control cells ($P < 0.05$). Fish oil treatment increased micro-diffusion of the FP receptors on luteal cells by 95% when compared to receptors from control luteal cells ($P < 0.05$; Fig 13A). Furthermore, fish oil treatment increased macro-diffusion of FP receptors by 166% when compared to control luteal cells ($P < 0.05$; Fig 13B).

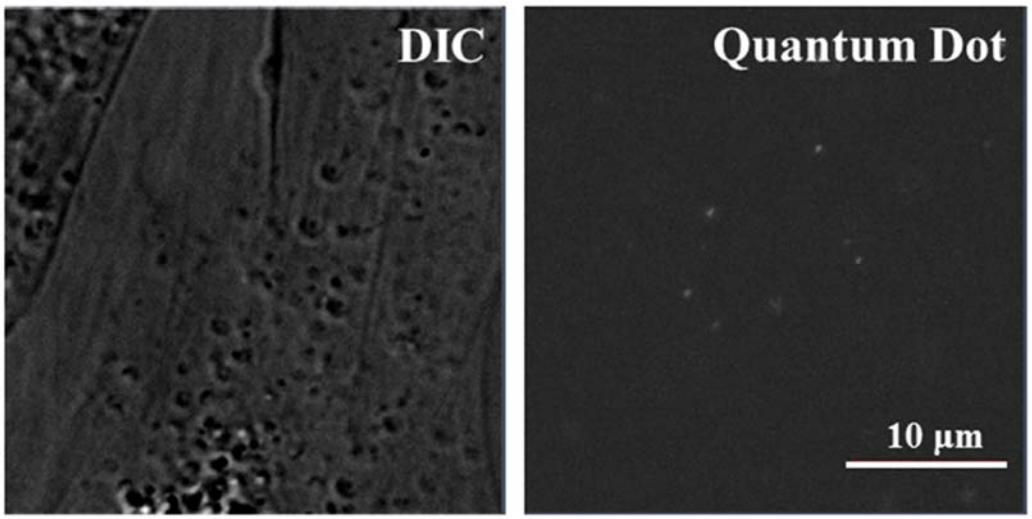

Figure 11. Verification of prostaglandin F_{2alpha} receptor labeling on bovine luteal cells. Representative micrograph of bovine luteal cells bound with biotin-conjugated prostaglandin $F_{2\alpha}$ FP antibody and quantum dots.

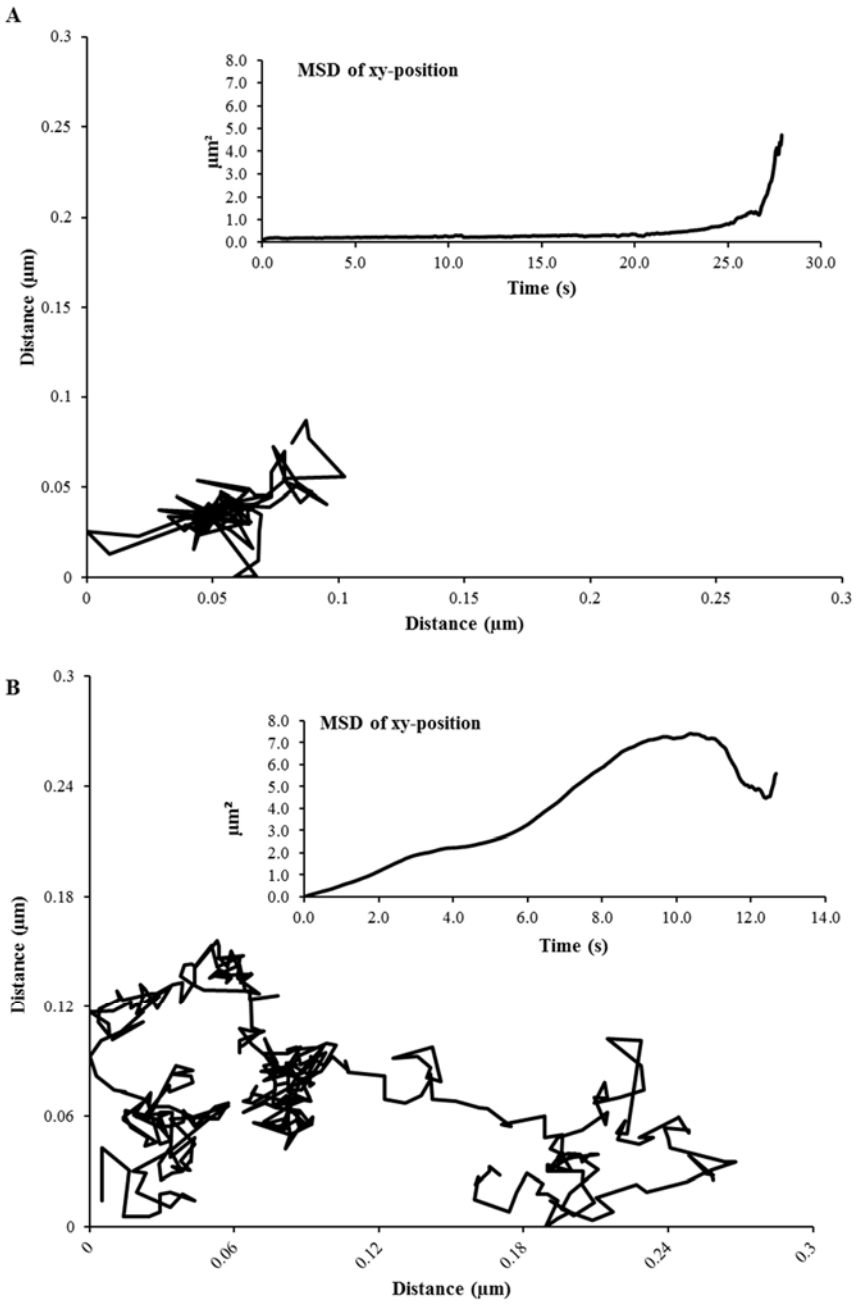

Figure 12. Trajectories of individual prostaglandin F_{2alpha} receptor on the plasma membrane of control and 0.03% (vol/vol) fish oil treated bovine luteal cells.
Individual trajectories of prostaglandin $F_{2\alpha}$ FP receptor on luteal cell membranes obtained from control (panel A) and 0.03% fish oil (panel B) treated cells. Inset represents corresponding mean square displacement of each trajectory which was calculated and plotted as a function of time.

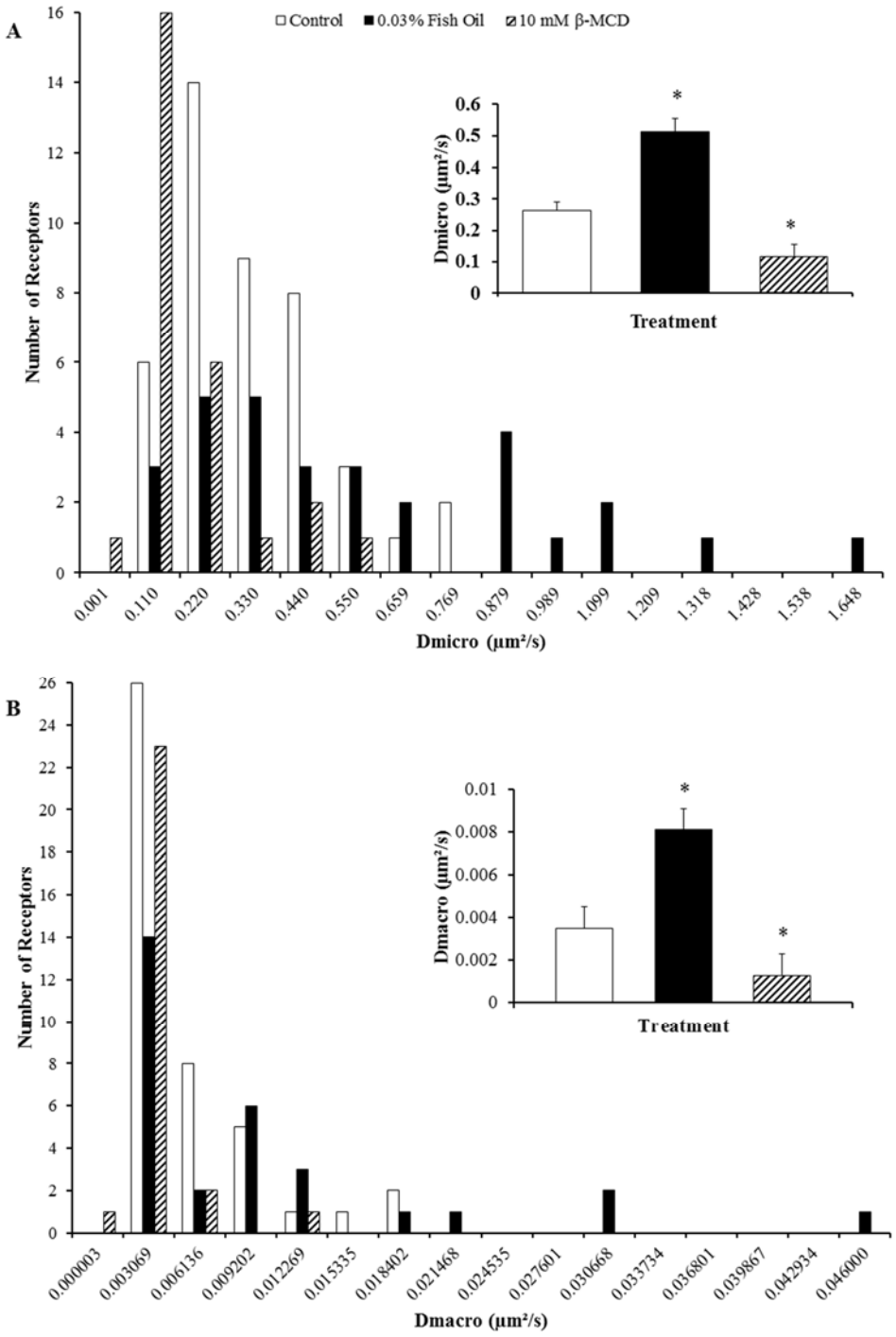

Figure 13. Effects of fish oil on lateral mobility of prostaglandin F_{2alpha} receptors on bovine luteal cells. (A) micro- and (B) macro-diffusion coefficient of the prostaglandin $F_{2\alpha}$ (FP) receptor on bovine luteal cells treated with control or 0.03% (vol/vol) fish oil treated cells. The inset in each panel shows the mean ± standard error of the mean * $P <$ 0.05. Control (n= 44 FP receptors), Fish oil 0.03% (vol/vol; n= 29 FP receptors), and 10 mM beta-methylcyclodextrin (β-MCD) (n= 26 FP receptors).

Fish oil treatment also affected domain size and the time a FP receptor resided within a domain, which is referred to as residence time and are shown in Figure 14. The residence time of FP receptors on luteal cells from 0.03% (vol/vol) fish oil treatment was decreased by 25% when compared to receptors on luteal cells from control treatment ($P < 0.05$; Fig 14A). Furthermore, fish oil treatment increased the domain size, or corral, of the microdomains by 36% ($P < 0.05$; Fig 14B).

Pre-treatment of luteal cells with β-MCD to remove cholesterol from the plasma membrane resulted in a 56% reduction in micro-diffusion as compared to receptors on luteal cells from control treatment ($P < 0.05$; Fig 13A). Furthermore, macro-diffusion was decreased by 66% when compared to control cells ($P < 0.05$; Fig 13B). The residence time of the FP receptors on luteal cells pre-treated with β-MCD was increased by 53% when compared to receptors on luteal cells from control treatment ($P < 0.05$; Fig 14A). Moreover, the depletion of cholesterol by β-MCD decreased the domain size of the microdomains by 35% when compared to control cells ($P < 0.05$; Fig 14B).

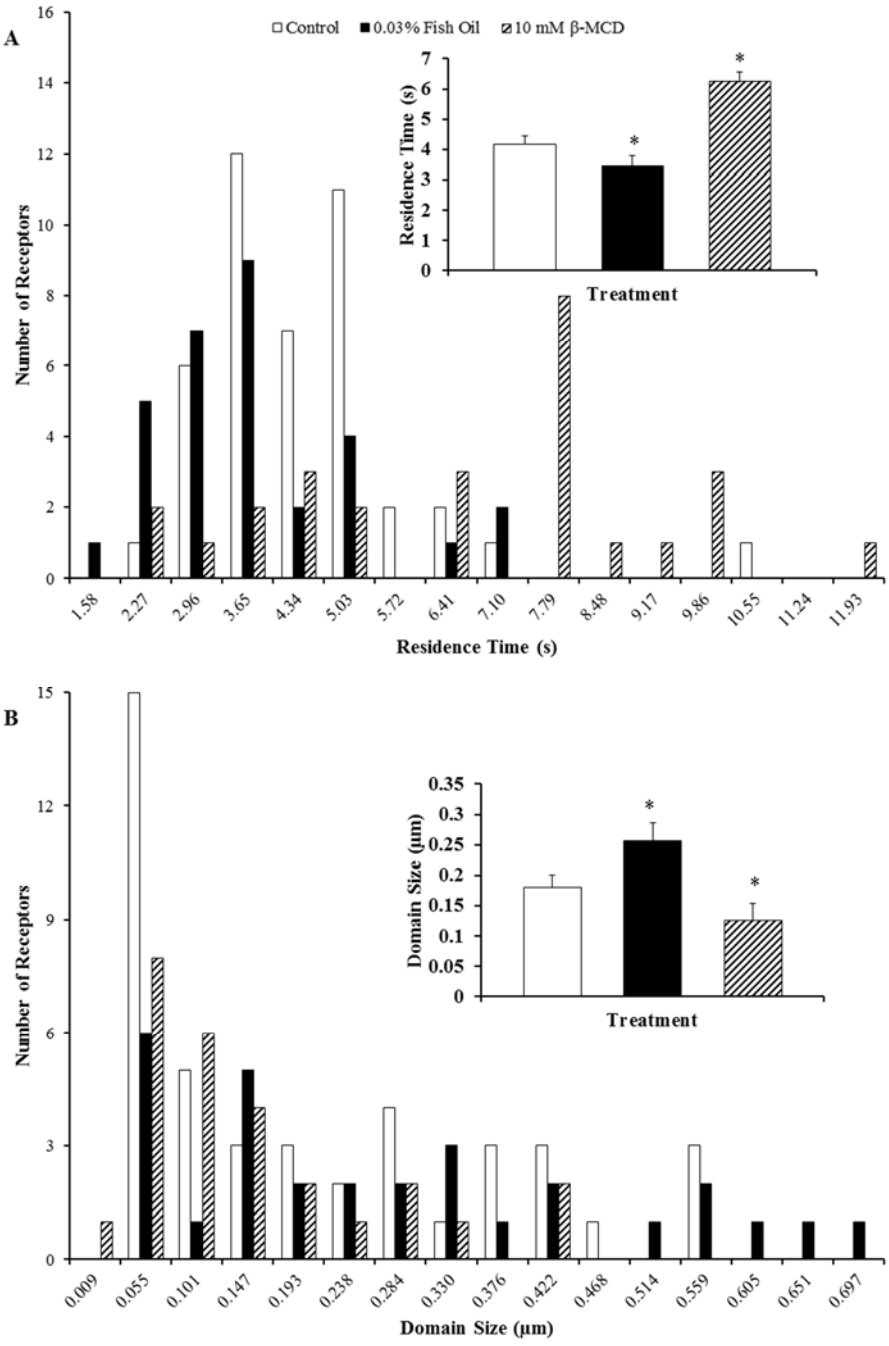

Figure 14. Effects of fish oil on domain size and residence time of prostaglandin F_{2alpha} receptors on bovine luteal cells. (A) residence time and (B) microdomain diameter of the prostaglandin $F_{2\alpha}$ (FP) receptors on bovine luteal cells treated with control, 0.03% (vol/vol) fish oil, or 10 mM beta-methylcyclodextrin (β-MCD) treated cells. The inset in each panel shows the mean ± standard error of the mean * $P < 0.05$. Control (n= 44 FP receptors), Fish oil 0.03% (vol/vol; n= 29 FP receptors), and 10 mM β –MCD (n= 26 FP receptors).

Experiment 3: Effects of Fish Oil on Lateral Mobility of Prostaglandin $F_{2\alpha}$ Receptors on Bovine Luteal Cells in the Presence of Prostaglandin $F_{2\alpha}$.

Effects of $PGF_{2\alpha}$ on micro-diffusion of FP receptors on bovine luteal cell membranes are shown in Figure 15A. In control luteal cells, there was no difference in micro-diffusion at 5 min after addition of ligand ($P > 0.10$). However, micro-diffusion of FP receptors was reduced at both 15 and 30 min after addition of ligand when compared prior to addition of ligand (0 min; $P < 0.05$). As in Experiment 2, micro-diffusion of FP receptors was greater for fish oil treated luteal cells before addition of ligand as compared to control luteal cells ($P < 0.05$). Unlike control luteal cells, the addition of $PGF_{2\alpha}$ had no effect on micro-diffusion of FP receptors at any of the time points for luteal cells treated with fish oil. As a result of ligand not affecting mobility of receptors, micro-diffusion of FP receptors were greater for fish oil treated cells a 5, 15, and 30 min post-treatment when compared to control luteal cells ($P < 0.05$). Depletion of cholesterol from the membrane using β-MCD decreased micro-diffusion of FP receptors when compared to control luteal cells as in Experiment 2 ($P < 0.05$). Micro-diffusion of FP receptors was unaffected by the addition of $PGF_{2\alpha}$ for luteal cells pre-treated with β-MCD ($P > 0.10$).

Effects of $PGF_{2\alpha}$ on macro-diffusion of FP receptors on bovine luteal cell membranes are shown in Figure 15B. In control luteal cells, there was no difference in macro-diffusion at 5 min after addition of ligand ($P > 0.10$). However, macro-diffusion of FP receptors was reduced at both 15 and 30 min after addition of ligand when compared prior to addition of ligand (0 min; $P < 0.05$). Macro-diffusion of FP receptors on fish oil treated luteal cells prior to the addition of ligand was increased by 198% as

compared to receptors on luteal cells from control treatment ($P < 0.05$). Macro diffusion was not affect by the addition of $PGF_{2\alpha}$ in luteal cells treated with fish oil or β-MCD ($P > 0.10$).

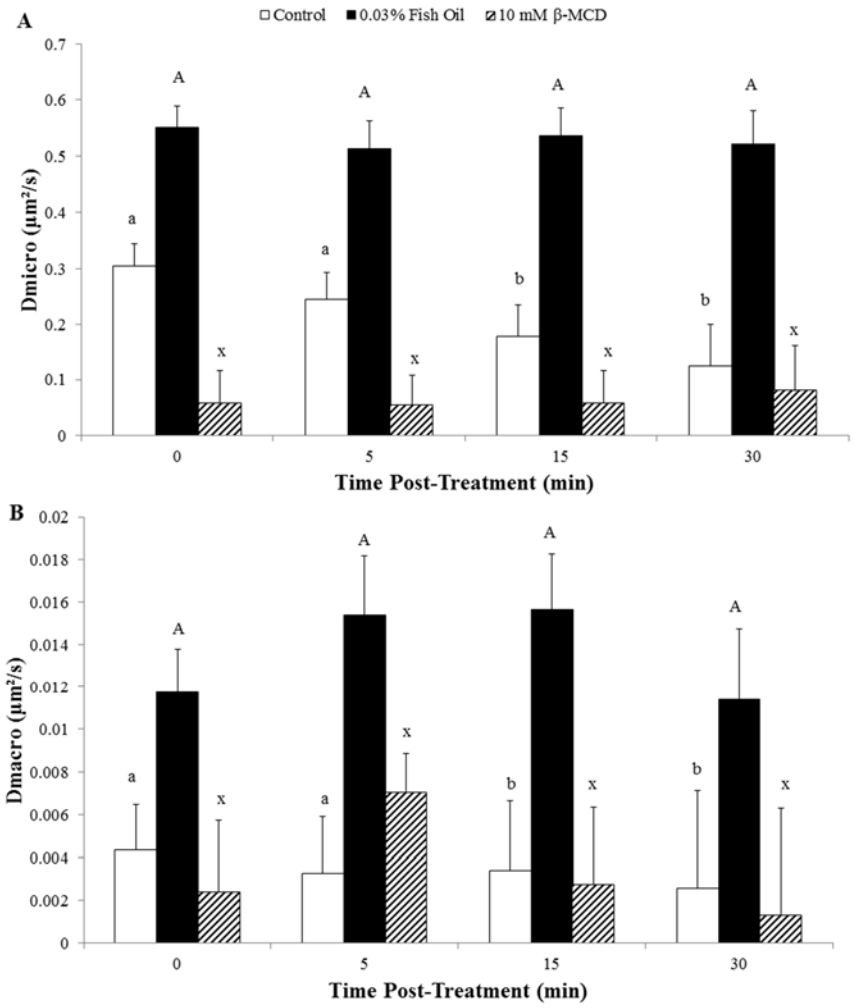

Figure 15. Effects of fish oil on lateral mobility of prostaglandin F_{2alpha} receptors in the presence of ligand on bovine luteal cells. (A) micro- and (B) macro-diffusion coefficient of the prostaglandin $F_{2\alpha}$ (FP) receptors on bovine luteal cells treated with control, 0.03% (vol/vol) fish oil, or 10 mM beta-methylcyclodextrin (β-MCD) treated cells in the presence of 10 mM prostaglandin $F_{2\alpha}$. Fish oil 0.03% (vol/vol). 10 mM β-MCD. ^{a,b,c} Control cells - means with different letters differ ($P < 0.05$) when compared to 0 min. ^{A,B,C} Fish oil cells - means with different letters differ ($P < 0.05$) when compared to 0 min.$^{x, y,z}$ β-MCD cells - means with different letters differ ($P < 0.05$) when compared to 0 min. Number of receptors analyzed at each time point. Control (n=80, 52, 31, and 14 FP receptors, respectively), Fish oil 0.03% (vol/vol; n=86, 48, 57, and 32 FP receptors, respectively), and 10 mM β–MCD (n=35, 26, 22, and 9 FP receptors, respectively)

Effects of $PGF_{2\alpha}$ on residence time of FP receptors in a domain are shown in Figure 16A. The addition of $PGF_{2\alpha}$ increased residence time of FP receptors in a domain for control luteal cells within 5 min after the addition of ligand ($P < 0.05$). Residence time of FP receptors in a domain was 20% greater for luteal cells treated with fish oil treatment when compared to control luteal cells before addition of $PGF_{2\alpha}$ ($P < 0.05$). Unlike control luteal cells, $PGF_{2\alpha}$ treatment had no effect on residence time of FP receptors at 5, 15, or 30 min post-treatment ($P > 0.10$). The residence time for FP receptors were greater for luteal cells treated with β-MCD before $PGF_{2\alpha}$ treatment when compared to control luteal cells ($P < 0.05$). The addition of $PGF_{2\alpha}$ further increased residence of FP receptors for luteal cells pre-treated with β-MCD ($P < 0.05$).

Effects of $PGF_{2\alpha}$ on domain size are shown in Figure 16B. Size of domain was influenced by fish oil treatment. Domains were larger when compared to control luteal cells ($P < 0.05$). Prostaglandin $F_{2\alpha}$ treatment did not affect domain size for control, fish oil treated, or pre-treated β-MCD luteal cells ($P > 0.10$).

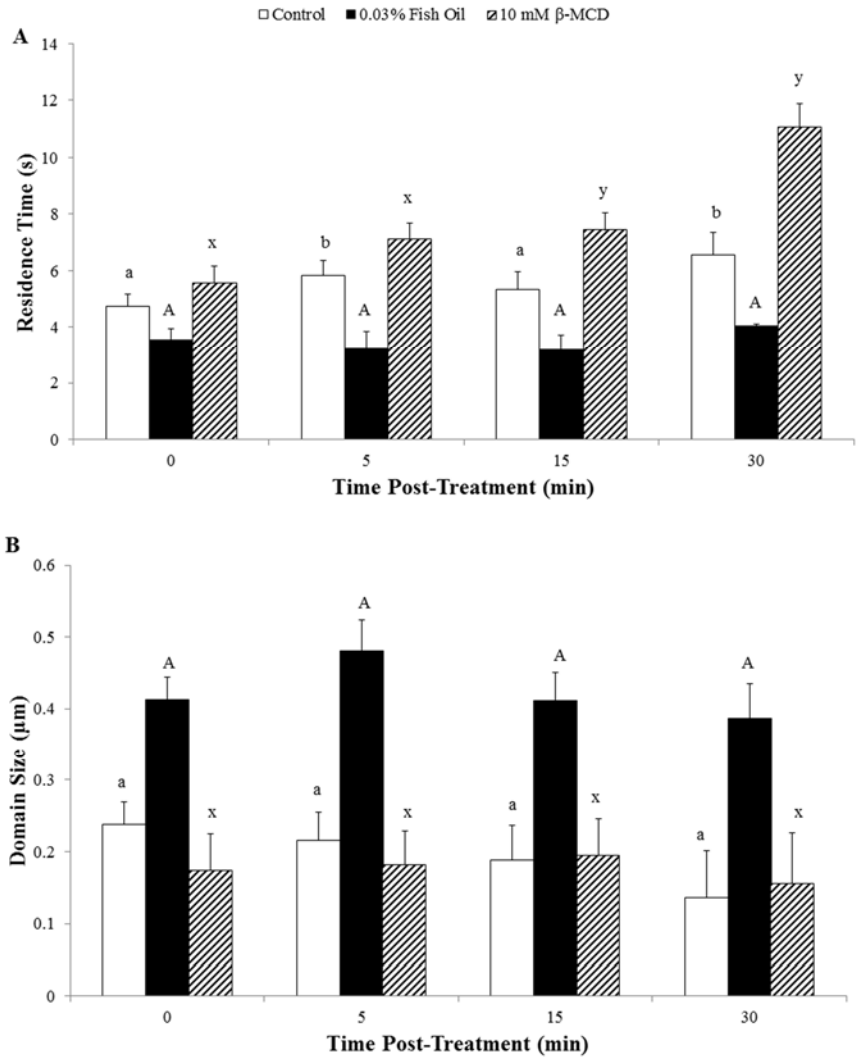

Figure 16. Effects of fish oil on domain size and residence time of prostaglandin F_{2alpha} receptors in the presence of ligand on bovine luteal cells. (A) residence time and (B) microdomain diameter of the prostaglandin $F_{2\alpha}$ (FP) receptors on bovine luteal control, 0.03% (vol/vol) fish oil, or 10 mM beta-methylcyclodextrin (β-MCD) treated cells, in the presence of 10 mM prostaglandin $F_{2\alpha}$. Fish oil 0.03% (vol/vol). 10 mM β-MCD. ^{a,b,c} Control cells - means with different letters differ ($P < 0.05$) when compared to 0 min. ^{A,B,C} Fish oil cells - means with different letters differ ($P < 0.05$) when compared to 0 min.$^{x, y,z}$ β-MCD cells - means with different letters differ ($P < 0.05$) when compared to 0 min. Number of receptors analyzed at each time point. Control (n=80, 52, 31, and 14 FP receptors, respectively), Fish oil 0.03% (vol/vol; n=86, 48, 57, and 32 FP receptors, respectively), and 10 mM β–MCD (n=35, 26, 22, and 9 FP receptors, respectively)

The decreased number of FP receptors visible on the plasma membrane for control, β-MCD, and 0.03% fish oil treated cells at 30 min post-treatment were 81 ± 6, 91 ± 6, and 37 ± 6%, respectively. This decrease in the percentage of visible receptors did not differ between control and β-MCD treated cells ($P > 0.10$). However, when compared to 0.03% fish oil treated cells, there was a decrease in the percentage of visible receptors for both control and β-MCD ($P < 0.05$).

Discussion

Biological membranes including the plasma membrane of cells are made of a bilayer of lipids with associated integral and peripheral proteins (92)]. The major lipids of membranes include glycerophospholipids, sterols, and sphingolipids (55-57)]. Once postulated to be homogeneous, the plasma membrane is asymmetric in regards to lipid composition and is very dynamic (93)]. Early studies in the 1970's using artificial membranes showed that the lipids of the bilayer segregate into lipid ordered states, now referred to as lipid microdomains (94)]. Lipid microdomains were detected as distinct patches on the plasma membrane of control luteal cells using cholera toxin binding to monosialotetrahexosylganglioside, a known marker of lipid microdomains, and antibody crosslinking. The present study shows that culturing bovine luteal cells in the presence of fish oil had a dramatic effect on lipid microdomain structure. Fish oil resulted in an increase in the spatial distribution of these domains in a dose-dependent manner. The results from this study are in agreement with previous observations that supplementation with fish oil, either *in vitro* or *in vivo*, affects the order of lipid microdomains on the plasma membrane (74, 95-97)].

The mechanism by which fish oil affects lipid microdomain structure is still largely unknown. It is postulated that long chain polyunsaturated fatty acids, specifically the omega-3 fatty acids eicosapentaenoate and docosahexaenoate, may play a key role in the disruption of the structural integrity of lipid microdomains. Generally, long-chain fatty acids associated with microdomains are saturated, allowing for tight packing, thereby increasing lipid order (75). The long chain omega-3 polyunsaturated fatty acids prevalent in fish oil can incorporate into the lipid microdomains (53, 74), which may increase the fluidity of this ordered region of the plasma membrane. Alteration of membrane fluidity or microdomain structure with fish oil may affect mobility of integral and peripheral proteins associated with the plasma membrane.

To our knowledge, this is the first study examining lateral mobility of FP receptors on the plasma membrane of bovine luteal cells using single particle tracking methodology. Receptors were successfully labeled and tracked and resulting trajectories from individual receptors allowed for the estimation of diffusion coefficients, domain sizes, and residence times. Micro-and macro-diffusion coefficients, domain size, and residence time of FP receptors obtained from control luteal cells were slightly greater than values reported in the literature for membrane-bound receptors. Most studies reported in the literature with lower diffusion coefficients for membrane-bound receptors were conducted using transfected cells (89, 98, 99)]. The introduction of membrane-bound receptors to the plasma membrane using transfection may lead to overexpression of receptors causing an increase in protein crowding. Increased protein crowding has been reported to decrease mobility of membrane proteins in simulated membrane models (100, 101)] and artificial membranes (102)] which may account for differences in

mobility characteristics in the present study and other studies using transfected cell lines. Moreover, the steroidogenic nature and potential increased cholesterol content within the plasma membrane of bovine luteal cells (103)] could play a role in the increased diffusion coefficients observed in this study as well. However, more importantly, luteal cells cultured in the presence of fish oil had increased lateral mobility of the FP receptor when compared with control luteal cells. Furthermore, the average time a receptor resided in a domain was decreased compared to receptors from controls. Likewise, increased domain sizes were observed with fish oil treated cells, confirming spatial distribution results, indicating increased structural disruption of lipid microdomains. Taken together, the disruption of spatial distribution of lipid microdomains and increased lateral mobility of the FP receptors indicate that fish oil supplementation could potentially influence sensitivity of FP receptors to $PGF_{2\alpha}$.

Effects of ligand on the lateral mobility of the FP receptor in bovine luteal cells were also examined in the present study. Lateral mobility of FP receptors (both micro- and macro-diffusion) in control luteal cells decreased following the addition of ligand. Furthermore, the number of visible receptors on the membrane decreased following addition of ligand. As previously discussed, lipid microdomains may play a role in co-localization of membrane-bound receptors with its associated intracellular signaling proteins. In addition to cell signaling, these domains may play a role in endocytosis of ligand-bound receptors, leading to desensitization to additional ligand (104)]. It is hypothesized that binding of $PGF_{2\alpha}$ to its receptor resulted in receptor docking within the lipid microdomains between 5 to 15 min post-treatment, allowing for downstream signaling. Within 30 min post-treatment, there was clearly a decrease in number of

receptors visible on the membrane, indicating possible endocytosis of ligand-bound FP receptors. Endocytosis of the FP receptor may be occurring through a negative feedback mechanism, which includes the phosphorylation of receptors by protein kinases (105)] and binding of β-arrestin, following the addition of ligand (106)]. However, unlike in control luteal cells, the lateral mobility of the FP receptor in fish oil treated cells remained unchanged during the 30 min of stimulation. Likewise, the number of visibly labeled receptors on the cell membrane 30 min post-treatment was higher than control luteal cells, possibly indicating a potential disruption in downstream cell signaling and (or) endocytosis of ligand-bound receptors.

The mechanism(s) by which fish oil affects lateral mobility of membrane-bound FP receptors on bovine luteal cells is unknown. The increased mobility most likely is due to greater membrane fluidity in both lipid microdomains and bulk lipid allowing receptors to diffuse at a greater rate. The increased domain size in response to fish oil may be due to the incorporation of omega-3 polyunsaturated fatty acids. The triglycerides in fish oil must be hydrolyzed to release the omega-3 polyunsaturated fatty acids prior to the incorporation of free eicosapentaenoate and docosahexaenoate into the plasma membrane of the luteal cells. It is however, possible that the triglycerides are hydrolyzed catalytically by the lipases present in the fetal bovine serum present in the media. In addition, it has been reported that intact triglycerides can diffuse through plasma membranes; thus the triglycerides may be hydrolyzed by luteal lipases (107). Experiments are ongoing in our laboratory investigating the influence of individual omega-3 polyunsaturated fatty acids on lipid microdomain structure and lateral mobility of FP receptors on bovine luteal cells.

In this study, β-MCD was used as a disruptor of lipid microdomains. Cholesterol content within the plasma membrane of bovine luteal cells also affected lipid microdomain structure, mobility of FP receptors, and residence time within domains which differed greatly from fish oil treated cells. These data are in agreement wherein cholesterol is critical for microdomain integrity (108)]. Removal of cholesterol from these domains can allow for the rearrangement of lipids (109)], membrane proteins (110)], or the cytoskeleton (111)]. This reorganization of the plasma membrane may lead to larger or disordered lipid microdomains. Further investigation of interaction of cholesterol with the cytoskeleton and lipids in microdomains is warranted in bovine luteal cells. Removal of cholesterol by β-MCD reduced lateral mobility of FP receptors and increased residence time. These data are in agreement with previous studies where acute depletion of cholesterol reduces mobility of membrane proteins and increases confinement (111, 112)].

The role of cholesterol in regulating lipid microdomain structure and mobility of lipids and proteins within the plasma membrane still needs defining. The hydrocarbon rings of cholesterol interact with the acyl chains of long-chain fatty acids esterified to glycerophospholipids that regulate plasma membrane fluidity. The concentration of β-MCD used in this study removes approximately 80% of the cholesterol (113)] which would allow for packing of acyl chains reducing fluidity of the membrane and most likely lateral mobility of the FP receptor. Acute depletion of cholesterol in the present study also increased residence time of FP receptors which has been shown in previous studies in which acute or chronic depletion of cholesterol increased confinement of membrane bound proteins (111, 112)]. Depletion of membrane cholesterol results in reorganization

of the cytoskeleton and phosphatidylinositol which may lead to increased confinement of membrane-bound receptors. A similar mechanism may be occurring with the FP receptor in cholesterol-depleted luteal cells and warrants further investigation.

Conclusion

The data presented here provide strong evidence that fish oil supplementation leads to disruption in lipid microdomains. This disruption resulted in increased spatial distribution of lipid microdomains and lateral mobility of the FP receptor. Furthermore, addition of $PGF_{2\alpha}$ restricted lateral mobility of FP receptors and increased residence time of receptors in domains in control cells, but had no effect on fish oil treated cells. This study showed that fish oil causes an increase in lateral mobility in the presence of ligand, indicating that fish oil supplementation could potentially influence sensitivity of FP receptors to $PGF_{2\alpha}$.

CHAPTER 3

EFFECT OF FISH OIL ON AGONIST-INDUCED MEDIATED RECEPTOR INTERNALIZATION OF THE PROSTAGLANDIN F2ALPHA (FP) RECEPTOR AND CELL SIGNALING IN BOVINELUTEAL CELLS *IN VITRO*

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Contribution of Authors and Co-Authors

Manuscript in Chapter III

Author: Michele R. Plewes

Contributions: Developed and implemented the study design. Generated and analyzed data. Wrote first draft of the manuscript.

Co-Author: Patrick. D. Burns

Contributions: Helped conceive the study topic. Provided guidance on study design. Provided feedback data interpretation and manuscript preparation.

Abstract

Many receptors span the plasma membrane allowing for signal transduction, converting extracellular signals into intracellular signals. Following ligand-induced activation, membrane-bound receptors are taken into endocytic vesicles, where they are targeted for degradation or recycled back to the plasma membrane. The objectives of the current study were to determine the influence of fish oil on 1) prostaglandin (PG) $F_{2\alpha}$–induced receptor internalization and trafficking of the $PGF_{2\alpha}$ (FP) receptor, 2) cytoskeletal structural integrity, and 3) $PGF_{2\alpha}$-induced mitogen-activated protein kinase (MAPK) signaling in bovine luteal cells. Bovine ovaries were obtained from a local abattoir and corpora lutea (CL; n = 4 to 6) were digested using collagenase. For all experiments, cells were incubated in either BSA or fish oil-supplemented media for 72 h to allow incorporation of omega-3 fatty acids into biological membranes. Confocal microscopy was used to determine the influence of fish oil on $PGF_{2\alpha}$–induced receptor internalization and trafficking of the FP receptor and cytoskeletal structural integrity. Additionally, western blotting was used to determine the effects of fish oil on $PGF_{2\alpha}$-induced MAPK signaling in bovine luteal cells. Results from the present study demonstrate that fish oil disrupts the colocalization of $G_{\alpha q}$ with both caveolae microdomains and FP receptor as well as PGF2a-induced MAPK signaling. This disruption of the FP receptor with the G-protein alpha subunit may be one mechanism by which a MAPK signaling is diminished following the addition of $PGF_{2\alpha}$. Furthermore, fish oil disrupts FP receptor internalization and endosomal protein trafficking without detectable changes in the cytoskeleton.

Introduction

The prostaglandin (PG) $F_{2\alpha}$ (FP) receptor is a G-protein-coupled receptor (GPCR), found on the plasma membrane of bovine luteal cells. In the non-pregnant cow, $PGF_{2\alpha}$ binds to the FP receptor, initiating signaling cascades which lead to regression of the corpus luteum (CL), and another chance for pregnancy. In the pregnant cow, the embryo releases a cytokine, interferon τ, which attenuates uterine release of $PGF_{2\alpha}$, thereby rescuing the CL. Early embryonic mortality often occurs when a viable embryo fails to effectively control $PGF_{2\alpha}$ secretion, resulting in regression of the CL and termination of pregnancy (10). Supplementation of omega-3 fatty acids has been reported to have beneficial effects on PG metabolism by suppressing oxytocin-induced uterine synthesis of $PGF_{2\alpha}$ (43), and decreasing luteal sensitivity to $PGF_{2\alpha}$ in cattle (114). However, the mechanism by which omega-3 fatty acids exert their beneficial effects is still largely unknown.

Transmembrane receptors span the plasma membrane converting extracellular messages into intracellular responses. G-protein-coupled receptors are the largest class of membrane-bound receptors, interacting with a diverse group of agonists. Following agonist-mediated activation, GPCRs are internalized into the cell and sorted in endosomes for degradation (76, 77) or recycling to the plasma membrane (78). Receptor internalization functions as a key mechanism for signal desensitization (79) but has also been reported to resensitize GPCRs (80), recycling receptors back to the membrane. The mechanism that regulates agonist-induced internalization has been well characterized for GPCR (68, 81-85). However, the mechanism that regulates both FP receptor internalization and sorting in reproductive cells is still unknown.

G-protein-coupled receptors have been shown to utilize both clathrin-dependent and clathrin-independent, agonist-mediated, receptor internalization. Clathrin-dependent internalization normally occurs at specialized sites of the plasma membrane, where clathrin-triskelion-coated vesicles are assembled, internalizing membrane-bound receptors for sorting. Caveolae-raft-mediated endocytosis is a clathrin-independent mechanism that uses lipid microdomains to internalize membrane receptors, including GPCRs, and cargo into the cell. Omega-3 polyunsaturated fatty acids are a distinct class of fatty acids that have been reported to affect the cellular cytoskeleton (86), possibly influencing the lateral mobility of clathrin-coated pits through the cortical actin network. Previous work from our laboratory demonstrated that omega-3 polyunsaturated fatty acids disrupt lipid microdomains (70, 115, 116), potentially influencing signaling and receptor internalization.

The omega-3 class of polyunsaturated fatty acids are essential fatty acids involved in numerous biological processes such as inflammation (44, 45), cell signaling (46), and protection against oxidative stress (47). Additionally, omega-3 polyunsaturated fatty acids have been reported to have major impacts on organization and function of biological membranes. These fatty acids can become incorporated into glycerophospholipids and sphingolipids, disrupting lipid microdomain structure (70, 116). Fish oil is a rich source of omega-3 polyunsaturated fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Using single particle tracking methodology, we have shown that fish oil increases the lateral mobility of the FP receptor and prevents receptor confinement following the addition of $PGF_{2\alpha}$ (70). In addition to increasing lateral mobility of the FP receptor, fish oil treated cells had 43%

more visible FP receptors on the plasma membrane following the addition of $PGF_{2\alpha}$, as compared to control cells. However, the mechanism by which fish oil increases lateral mobility of the FP receptor and decreases possible receptor internalization in bovine luteal cells is largely unknown. We hypothesize that polyunsaturated fatty acids from fish oil incorporate into the plasma membrane leading to disruption in protein-protein interaction with the FP receptor and heterotrimeric G-protein, receptor internalization, and endosomal trafficking, ultimately leading to attenuated cell signaling in bovine luteal cells. The objectives of the current study were to determine the influence of fish oil on 1) $PGF_{2\alpha}$ -induced receptor internalization and endosomal trafficking of the FP receptor, 2) cytoskeletal structural integrity, 3) $PGF_{2\alpha}$-induced mitogen-activated protein kinase signaling in bovine luteal cells.

Methods

Tissue Collection, Cell Preparation, and Cell Culture

Bovine ovaries containing a mature CL (n = 4 to 6) were collected at a local abattoir and transported to the laboratory at the University of Northern Colorado in 1× sterile PBS. Tissue collection, cell preparation and cell culture were performed as previously described from our laboratory (70, 115, 116).

Fish Oil Preparation for In Vitro Culture

A commercial fish oil was used for all experiments (Pharmavite, Mission Hills, CA). Lipids in fish oil were pre-bound to BSA prior to the addition to culture. In brief, 0.03% (vol/vol) fish oil was added to culture medium containing 33 mg/mL fatty acid-free BSA as described by Mattos et al. (88) Control medium was prepared using the same conditions as fish oil treatment, without the addition of fish oil.

Effects of Fish Oil on Prostaglandin $F_{2\alpha}$ Receptor Internalization in Bovine Luteal Cells

Biotinylation of prostaglandin $F_{2\alpha}$ receptor. Polyclonal FP receptor antibody (Cayman Chemical, Ann Arbor, MI, USA) was conjugated to biotin as previously described and validated in our laboratory (70).

Treating and labeling cells for colocalization. Sterile No. 1 glass coverslips (22 × 22 mm) were individually placed in each well of a 6-well culture dish. Mixed luteal cell cultures were prepared from six CL and seeded 5×10^4 cells/well. Cells were cultured in control BSA medium or medium supplemented with fish oil for 72 h, at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. Following 72 h incubation, cells were then prepared for colocalization.

Cells were incubated with appropriate medium (BSA or fish oil) containing 10 nM $PGF_{2\alpha}$ analog (cloprostenol sodium, Merck Animal Health, Madison, NJ, USA) for 0, 5, 15, 30, 60, or 120 min. Incubations were terminated, and cells were rinsed 3 × with 1 mL 1× PBS. Cells were then fixed with 200 µL of 4% paraformaldehyde and incubated at 4 °C for 30 mins. Cells were rinsed 3 × with 1 mL 1× PBS following fixation. Cells were then incubated with 200 µL of 1% Triton-X in 1× PBS at room temperature for 10 min to permeabilize the membranes. Cells were then rinsed 3 × with 1 mL 1× PBS. Cells were then blocked in 5% normal goat serum for 1 h at room temperature. Cells were then rinsed 3 × with 1 mL 1× PBS.

Biotinylated FP receptor antibody (2 µg) and appropriate antibody for colocalization (Table 1) was added to each 6-well culture dish and incubated at 4 °C for 24 h. Following incubation, cells were rinsed 3 × with 1 mL 1× PBS to remove unbound

antibody. Cells were then incubated with appropriate secondary antibodies (Table 1) at room temperature for 60 min. Cells were rinsed 3 × with 1 mL 1× PBS to remove unbound antibody. Following labeling with antibodies, coverslips containing labeled cells were mounted to cleaned glass microscope slides using 10 µL SlowFade-Gold anti-fade mounting medium containing DAPI (Life Technologies; S36938). Coverslips were sealed to glass microscope slides using clear nail polish and placed in -22 °C until imaging.

Table 1: Characteristics of antibodies used for microscopy and western blotting

Antibody name	Dilution ratio	Species specificity	Source	Cat. No
FP Receptor	1:50	Bovine	Rabbit pAB	*101802*
Caveolin-1[1]	1:50	Bovine	Rabbit mAB	3267
Clathrin	1:50	Mouse	Rabbit mAB	4796
EEA1	1:50	Mouse	Rabbit mAB	3288
Rab5	1:50	Mouse	Rabbit mAB	3547
Rab7	1:50	Mouse	Rabbit mAB	9367
Rab11	1:50	Mouse	Rabbit mAB	5589
$G_{\alpha\ q/11}$	1:50	Bovine	Rabbit pAB	SC392
Caveolin-1[2]	1:50	Human	Mouse mAB	SC53564
MAPK 1/3 (ERK)	1:500	Mouse	Rabbit pAB	SC94
MAPK 1/3 (p-ERK)	1:500	Mouse	Mouse mAB	SC7382
MAPK 14 (p38)	1:500	Mouse	Rabbit pAB	SC535
MAPK 14 (p-p38)	1:500	Mouse	Rabbit pAB	SC7975R
MAPK 8/9 (JNK)	1:500	Mouse	Rabbit pAB	SC474
MAPK 8/9 (p-JNK)	1:500	Mouse	Mouse mAB	SC293136
Beta-Actin[3]	1:500	Bovine	Rabbit pAB	ab8227
Alpha-tubulin	1:50	Bovine	Mouse mAB	DM1A
HRP-linked	1:2000	Anti-rabbit		7074
HRP-linked	1:2000	Anti-mouse		7076
Alexa Fluor 350	1:200	Anti-mouse		A11045
Alexa Fluor 488	1:500	Anti-mouse		A11001
Alexa Fluor 568	1:500	Anti-rabbit		A1101011
Alexa Fluor 647	1:500	Anti-biotin		405237
Phalloidin- 488[4]	1:33		*Amanita phalloides*	A12379

[1]Caveolin-1 Anti-Rabbit was used to label cells for colocalization with FP Receptor
[2]Caveolin-1 Anti-Mouse was used to label cells for colocalization with $G_{\alpha\ q/11}$
[3]Beta actin used for western blot loading control
Phalloidin-488 was used to stain F-actin for immunofluorescence microscopy
FP Receptor, prostaglandin $F_{2\alpha}$ receptor; Clathrin, Clathrin Heavy Chain;
EEA1, Early Endosome Antigen 1; Rab5, Ras-related protein Rab-5A;
Rab7, Ras-related protein Rab-7, Rab11, Ras-related protein Rab-11A

Microscopy and analysis. Images were collected using a Zeiss confocal microscope equipped with a 40× water immersion objective (1.2 N.A) and acquisition image size of 512 × 512 pixel (33.3 μm × 33.3 μm). The appropriate filters were used to excite each fluorophore and emission of light was collected between 450 to 1000 nm. Approximately 30 cells were randomly selected from each slide for analysis. The JACoP plug-in was used in Image J software to determine the Manders' overlap coefficient for each image. The ratio of the summed pixel intensities from the green channel, for which the intensity of the red channel is greater than zero and below total intensity of the green channel, was defined as M1. Manders' coefficient, M1, was an indicator of the proportion of the green signal corresponding with signal in the red channel over its total intensity. Manders' M1 coefficient ranged from 0 to 1, which was then transformed into percent colocalization by multiplying M1 by 100.

Effects of Fish Oil on Cytoskeleton Integrity in Bovine Luteal Cells

Mixed luteal cell cultures were prepared from five CL and cultured in control medium or medium supplemented with fish oil as described in 2.3.2. Cells were fixed with 200 μL 4% paraformaldehyde, rinsed 3 × with 1 mL 1× PBS, and stained with phalloidin or anti-mouse alpha-tubulin (Table 1; Cell Signaling Technologies, Danvers, MA, USA) for 1 h at room temperature. Cells were rinsed 3 × with 1 mL 1× PBS to remove unbound stain. Cells were then incubated with anti-mouse secondary antibody (Table 1) for 1 h at room temperature. Cells were rinsed 3 × with 1 mL 1× PBS and mounted to glass microscope slides and visualized on a confocal microscope as describe in 2.3.2. Images were individually visualized for morphological changes of F-actin or alpha-tubulin within the cell.

Effects of Fish Oil on Prostaglandin $F_{2\alpha}$ Induced Mitogen-Activated Protein Kinase Signaling in Bovine Luteal Cells

Cell preparation, treatment, and quantitation of protein. Mixed luteal cell cultures were prepared from four CL and plated in T-25 culture flasks at 5×10^5 cells/flask. Cells were cultured in control BSA medium or medium supplemented with fish oil for 72 h, at 37 °C in an atmosphere of 95% humidified air and 5% CO_2, as described above. Cells were treated with medium alone, 100 ng/mL phorbol 12,13-dibutyrate ester (PDBu), or 10 nM $PGF_{2\alpha}$ for 0 or 15 min, at 37 °C in atmosphere of 95% air and 5% CO_2. Phorbol ester was dissolved in dimethyl sulfoxide (DMSO) and added to a final concentration of 0.04 % DMSO. Corresponding control and $PGF_{2\alpha}$ media were adjusted with an equal concentration of DMSO. The vehicle for $PGF_{2\alpha}$ was PBS and corresponding control and PdBu media were adjusted with an equal volume of PBS.

Following incubation, cells were immediately placed on ice and rinsed 3 × with 1 mL 1× PBS to remove excess media. Cells were removed from T-25 culture flask using a cell scrapper and the cell suspension was placed in a 1.7 mL microcentrifuge tube. Cells were centrifuged at 4 °C at 500 × *g* for 5 mins and the supernatant was removed from cell pellet. Cells were resuspended in 1 mL ice cold RIPA buffer containing 10 µL protease inhibitor (APExBIO, Houston, TX, USA) and 1 mM sodium orthovanadate. Cells were then homogenized using a hand-held homogenizer. Cells were centrifuged at 4 °C at 12,000 × *g* for 5 mins and the supernatant was removed and placed into new 1.7 mL microcentrifuge tube. Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA) was performed per manufacturer's protocol using microtiter plates. Samples were resuspended in 5 × Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5%

β-mercaptoethanol, 0.01% bromophenol blue) and plated on a dry heat bath at 100 °C for 6 min.

Western blotting analysis. Proteins were resolved using 10% SDS-PAGE. Protein samples (30 µg) were loaded into wells of the gel and a constant voltage of 140 V was applied for approximately 60 to 90 min to separate proteins. Proteins were then electrophoretically transferred to polyvinylidene difluoride membranes at 25 V for 90 min. Transfer efficiency of proteins to the membranes were evaluated by Ponceau S staining prior to blocking. Membranes were blocked with TBS-T (10 mM Tris-HCl pH 7.4, 140 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk solution at room temperature for 1 h. Membranes were incubated in primary antibody (Table 1) for 24 h at 4 °C for detection of total and phosphorylated MAPK. Membranes were rinsed 3 × with 1× TBS-T for 5 min. Membranes were then incubated with appropriate horse radish peroxidase-linked secondary antibody (Table 1) for 1 h at room temperature. Blots were then rinsed 3 × with 1× TBS-T for 5 min each. Chemiluminescent substrate (SuperSignal; Thermo Fisher Scientific) was applied per manufacturer's instructions. Blots were visualized using VersaDoc imaging system (Bio-Rad, Hercules, CA, USA) and the percent abundance of immunoreactive protein was determined using densitometry analysis in ImageJ.

Total MAPKs were normalized to beta-actin prior to calculation of fold induction. In brief, each beta-actin densitometry abundance was divided by the lowest beta-actin densitometry abundance to normalize beta-actin. Total MAPKs protein were normalized to individual beta-actin by dividing total protein and beta-actin. Phosphorylated MAPK was determined for each treatment and time point by dividing densitometry for

phosphorylated MAPK over total MAPK. Fold induction within treatment (control, $PGF_{2\alpha}$, and phorbol ester) was then calculated by dividing phosphorylated MAPK at 15 min by 0 min normalized densitometry. Fold induction was then determined for each supplemented media (control or fish oil) by dividing phosphorylated MAPK by control of each media fold increase at 0 and 15 min, for each treatment.

Statistical Analysis

All data are reported as least square means ± standard error of the mean and significance was declared at $P < 0.05$. The effects of fish oil on prostaglandin $F_{2\alpha}$ receptor internalization were analyzed using one-way analysis of variance. The statistical model included CL, media (BSA or fish oil), time, image, and residual error. Image was used as random variable in the model. Calculations were made using the mixed-model procedure of SAS. If main effects or interactions were significant, then means were separated using pre-planned t-tests and PDIFF option of SAS. Effects of fish oil on MAPK signaling were analyzed using two-way ANOVA. The statistical model included CL, media (BSA or fish oil), treatment (medium alone, PDBu, or $PGF_{2\alpha}$), time, protein, and residual error. Corpus luteum was used as random variable in the model. Calculations were made using the mixed-model procedure of SAS. If main effects or interactions were significant, then means were separated using pre-planned t-tests and PDIFF option of SAS.

Results

Effects of Fish Oil on Prostaglandin $F_{2\alpha}$ Receptor Internalization in Bovine Luteal Cells

G-protein subunit $G_{\alpha q}$ has been reported to be predominantly located within the caveolae lipid microdomains (67). Percent colocalization of $G_{\alpha q}$ with caveolin-1

increased 5 min following the addition of $PGF_{2\alpha}$ for control-treated cells ($P < 0.05$; FIG 17A and 17B; Appendix B), and 5 min for fish oil-treated cells ($P < 0.05$; FIG 17A and 17B; Appendix C). Additionally, percent colocalization of $G_{\alpha q}$ with caveolin-1 was lower in fish oil-treated cells at 0 and 5 min post-$PGF_{2\alpha}$, when compared to control-treated cells ($P < 0.05$; FIG 17A and 17B; Appendix C).

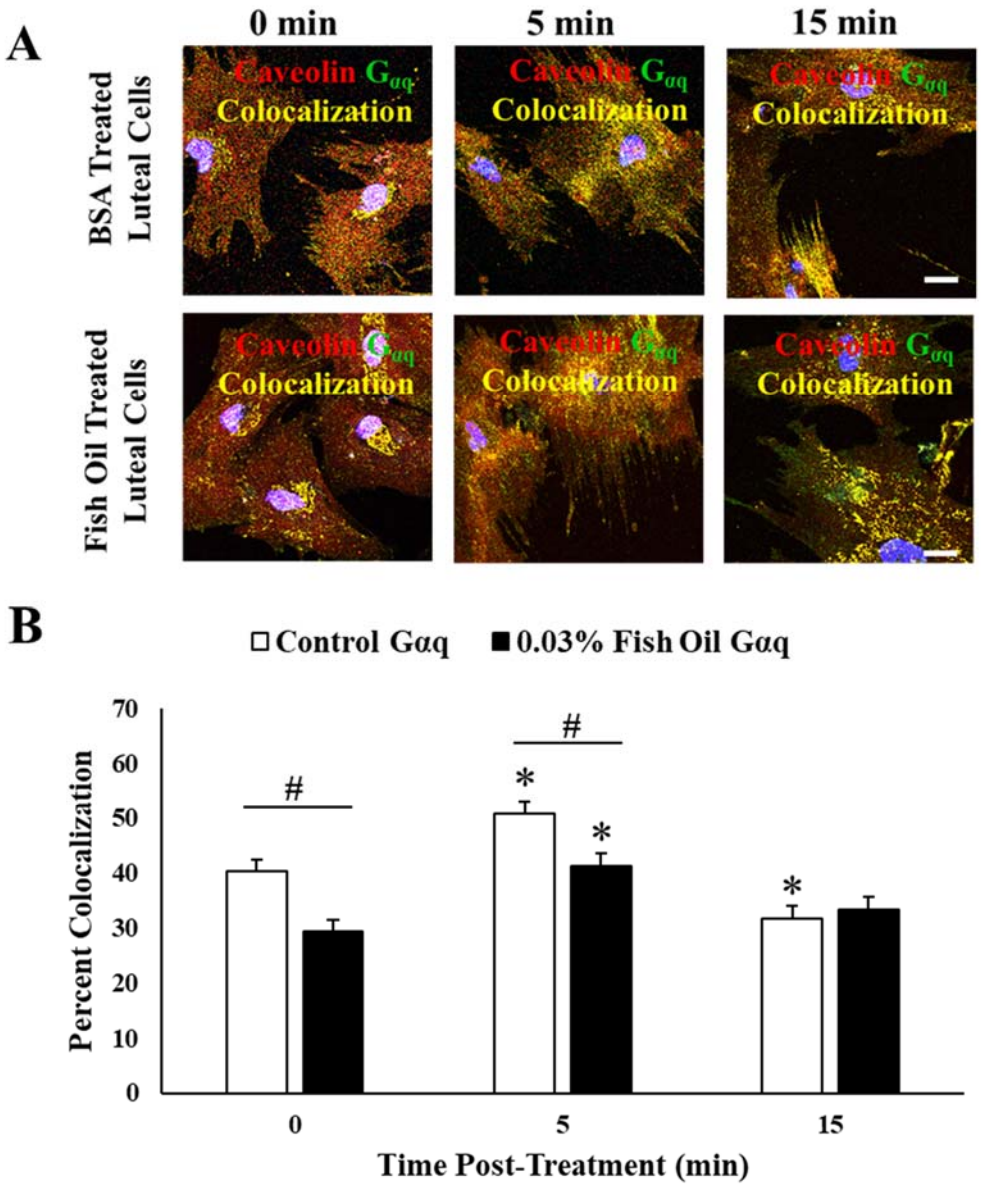

Figure 17. Effects of fish oil on colocalization of G_{alphaq} with caveolin. Panel A shows representative micrographs of the colocalization of caveolin with $G_{\alpha q}$ obtained from cells treated with BSA (A-C), or 0.03% (vol/vol) fish oil (D-F) following treatment with prostaglandin (PG) $F_{2\alpha}$. Panel B shows mean colocalization of $G_{\alpha q}$ with caveolin for luteal cells obtained from cells treated with BSA (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar). *Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. #Significant difference within time, $P < 0.05$. Micron bar represents 20 µm.

Percent colocalization of $G_{\alpha q}$ with FP receptor increased at 15 min following the addition of $PGF_{2\alpha}$ for control-treated cells ($P < 0.05$; FIG 18A and 18B; Appendix B). There was no difference in percent colocalization of $G_{\alpha q}$ with FP receptor in cells treated

with fish oil following the addition of $PGF_{2\alpha}$ ($P > 0.05$; FIG 18A and 18B; Appendix C). Additionally, percent colocalization of $G_{\alpha q}$ with FP receptor was lower in fish oil-treated cells at 0 and 5 min post-$PGF_{2\alpha}$, when compared to control-treated cells ($P < 0.05$; FIG 18A and 18B; Appendix C).

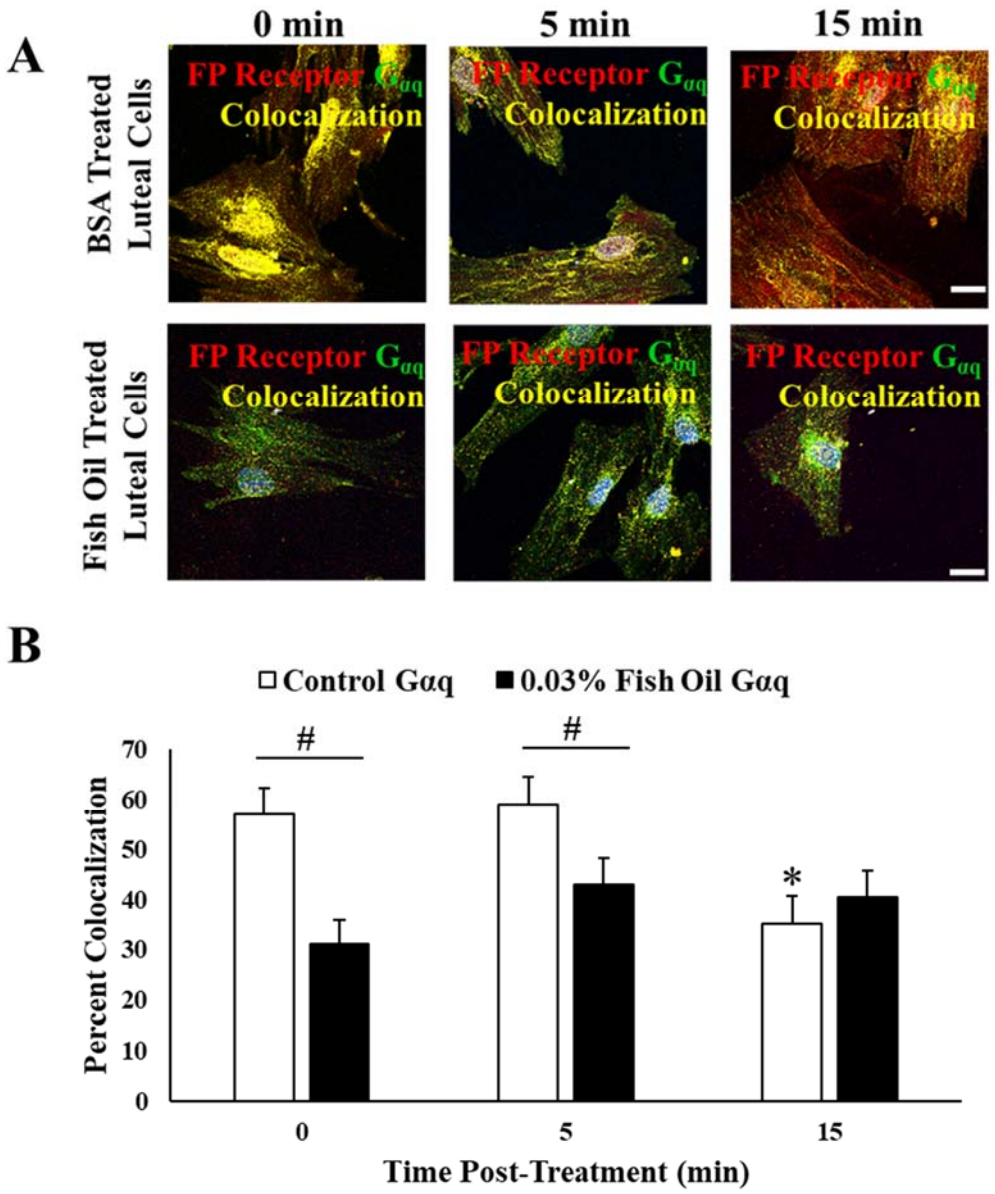

Figure 18. Effects of fish oil on colocalization of G_{alphaq} with the prostaglandin F_{2alpha} receptor. Panel A shows representative micrographs of the colocalization of prostaglandin $F_{2\alpha}$ (FP) receptor with $G_{\alpha q}$ obtained from cells treated with BSA (A-C), or 0.03% fish oil (D-F) following treatment with $PGF_{2\alpha}$. Panel B shows mean colocalization of $G_{\alpha q}$ with FP receptor for luteal cells obtained from cells treated with BSA (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar). *Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. $^{\#}$Significant difference within time, $P < 0.05$. Micron bar represents 20 µm.

Receptor internalization can be mediated through clathrin-dependent or -independent endocytosis. Caveolin-1 was used to determine if the FP receptor was mediated through clathrin-independent internalization. There was no effect of $PGF_{2\alpha}$ throughout all time points on percent colocalization of caveolin-1 with the FP receptor for both control and fish oil-treated luteal cells ($P > 0.05$; FIG 19A and FIG 19B; Appendix D and E). However, there was an increase in percent colocalization for clathrin and the FP receptor at 15 min post- $PGF_{2\alpha}$ for both control and fish oil-treated cells ($P < 0.05$; FIG 20A and FIG 20B; Appendix F and G), but no significant differences between the two supplementation groups at each time point.

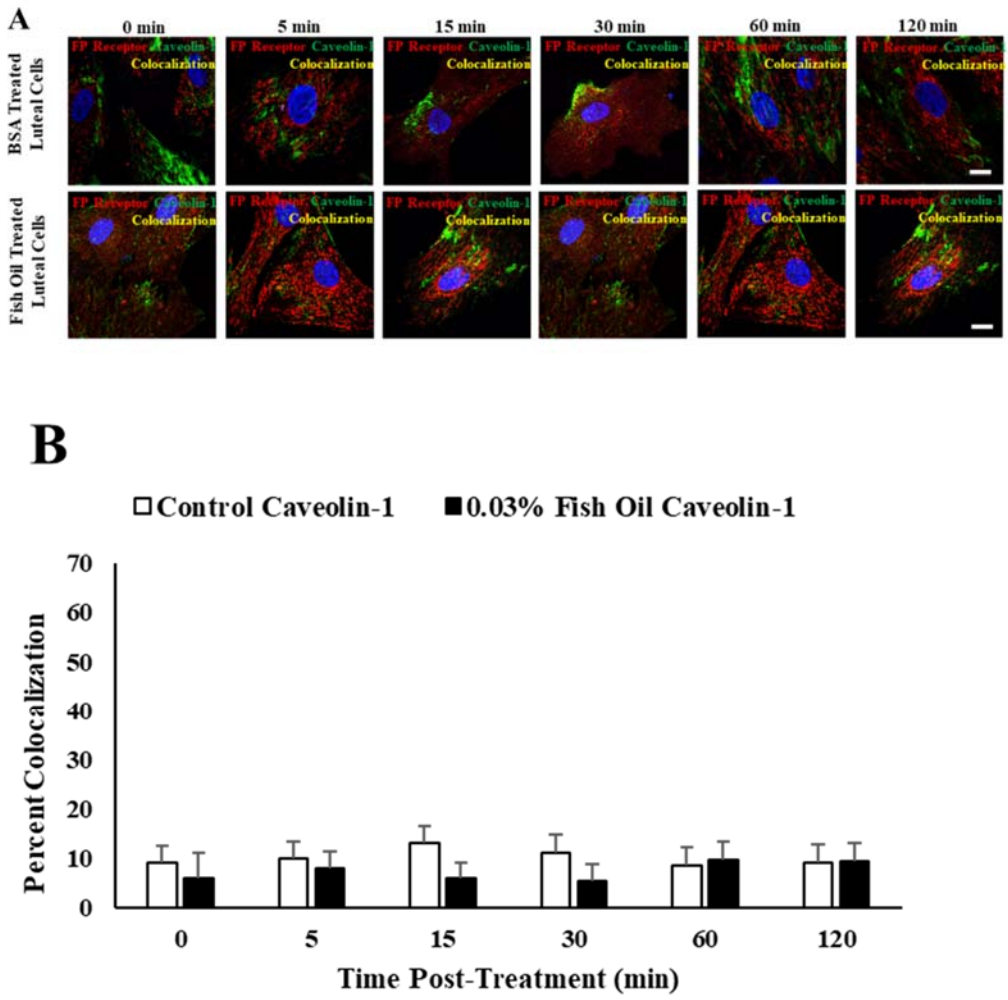

Figure 19. Effects of fish oil on prostaglandin F_{2alpha} receptor clathrin-independent internalization. Panel A shows representative micrographs of the colocalization of prostaglandin $F_{2\alpha}$ (FP) receptor with caveolin obtained from cells treated with BSA (A-F), or 0.03% (vol/vol) fish oil (G-L) following treatment with prostaglandin (PG) $F_{2\alpha}$. Panel B shows mean colocalization of FP receptor with caveolin for luteal cells obtained from BSA control (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar) cells. * Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. Micron bar represents 20 μm.

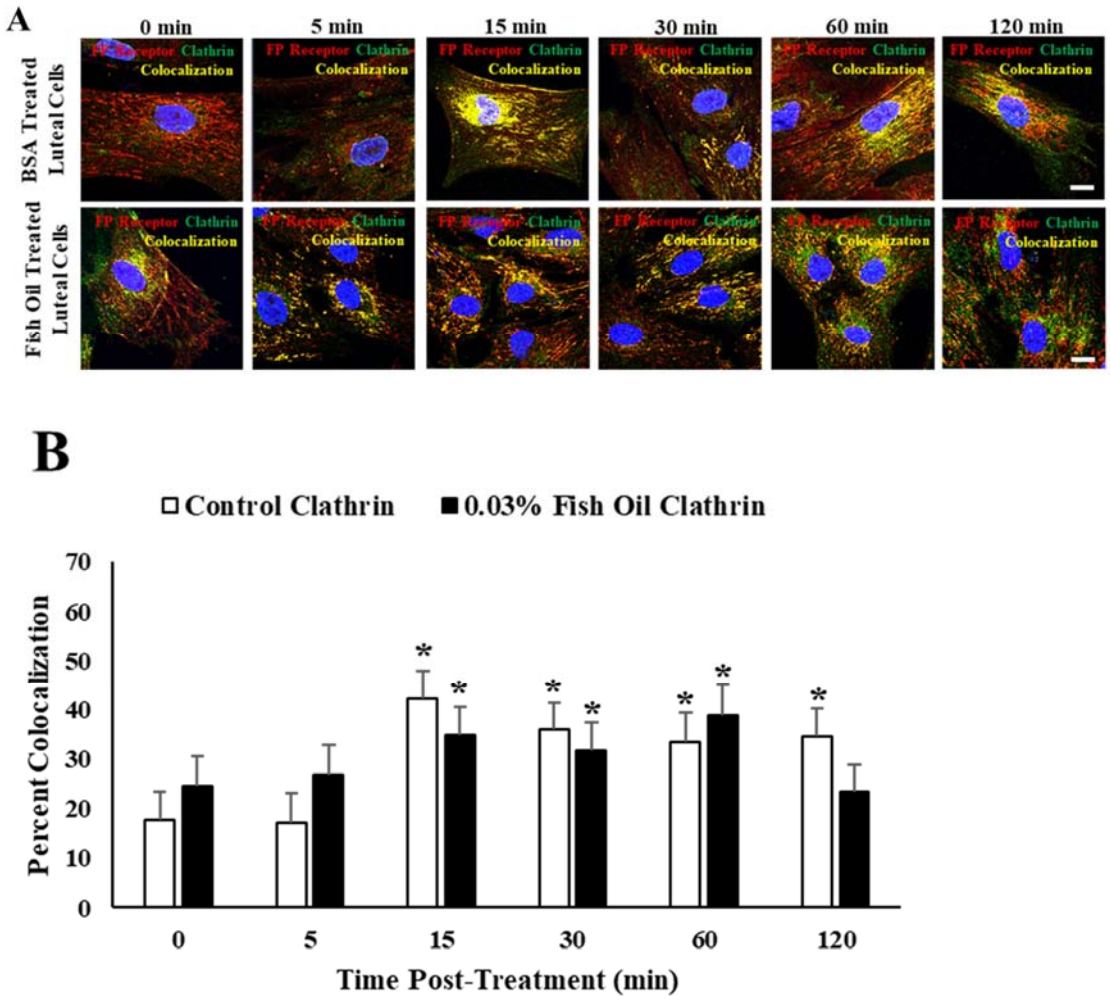

Figure 20. Effects of fish oil on prostaglandin F_{2alpha} receptor on clathrin-dependent internalization. Panel A shows representative micrographs of the colocalization of FP receptor with clathrin obtained from cells treated with BSA (A-F) or 0.03% fish oil (G-L), following $PGF_{2\alpha}$ treatment. Panel B shows mean colocalization of FP receptor with clathrin for luteal cells obtained from cells treated with BSA (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar). * Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. Micron bar represents 20 µm.

Colocalization with early endosome markers, EEA1 and Rab5a, was used to determine if the FP receptor was trafficked to early endosome following internalization. There was an increase in colocalization with EEA1 and the FP receptor at 5, 15, and 60 min post- $PGF_{2\alpha}$ treatment for control cells when compared to 0 min ($P < 0.05$; FIG 21A and 21B; Appendix H). Additionally, there was an increase in percent colocalization with

Rab5a at 5, 15, 30, and 120 min post- $PGF_{2\alpha}$ treatment for control cells when compared to 0 min ($P < 0.05$; FIG 22A and FIG 22B; Appendix J). There was no difference in percent colocalization of the FP receptor with either early endosome markers, EEA1 or Rab5a, for fish oil-treated cells ($P > 0.05$; FIG 21A and 21B; FIG 22A and 22B; Appendix I and K).

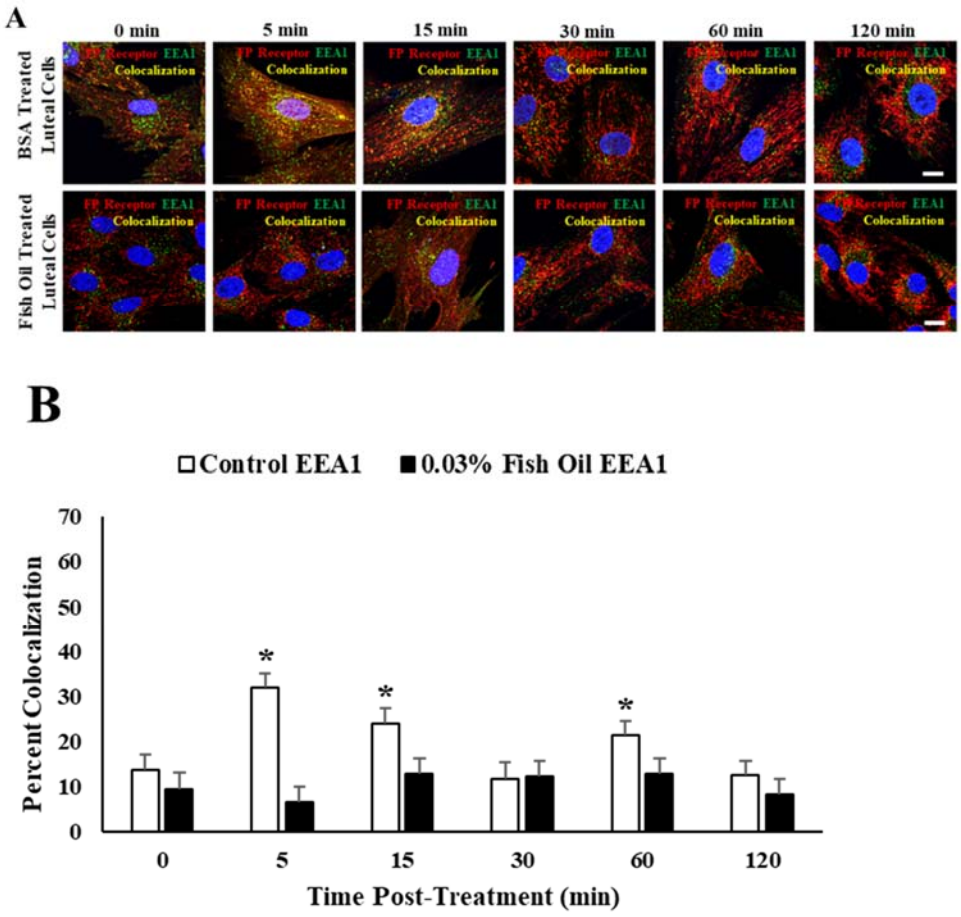

Figure 21. Effects of fish oil on prostaglandin F_{2alpha} receptor trafficking to early endosome associated protein. Panel A shows representative micrographs of the colocalization of prostaglandin $F_{2\alpha}$ (FP) receptor with EEA1 obtained from cells treated with BSA (A-F), or 0.03% (vol/vol) fish oil (G-L) following treatment with prostaglandin (PG) $F_{2\alpha}$. Panel B shows mean colocalization of FP receptor with EEA1 for luteal cells obtained from BSA control (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar) cells. *Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. Micron bar represents 20 µm.

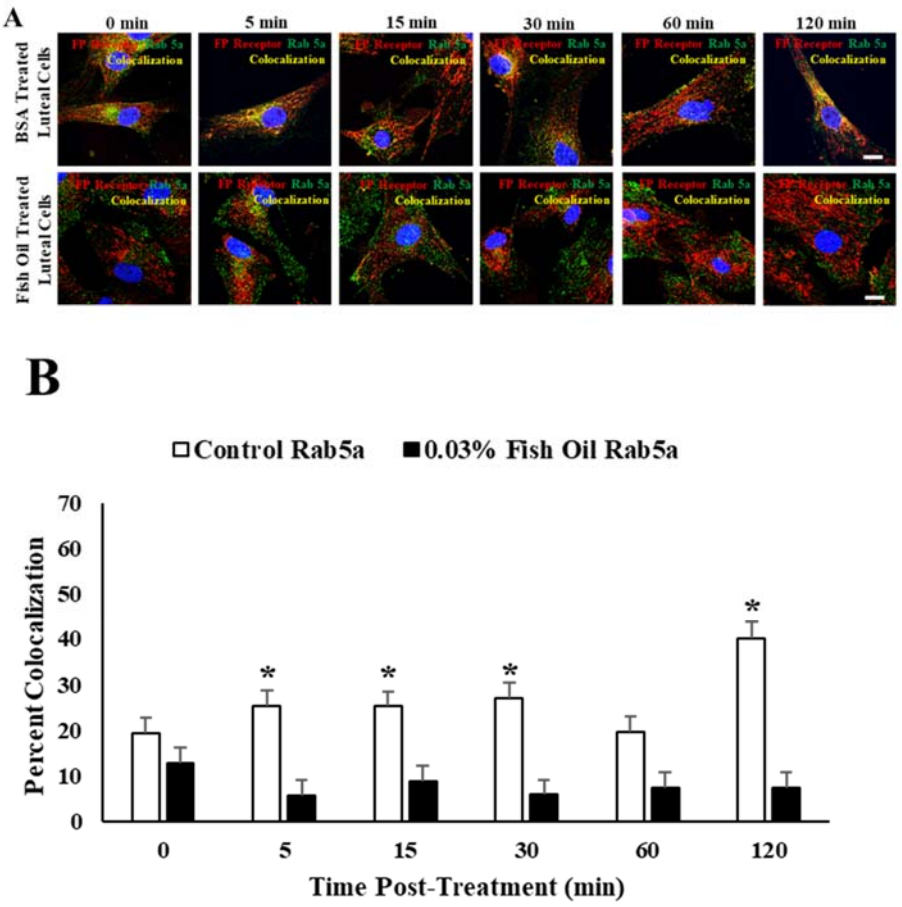

Figure 22. Effects of fish oil on prostaglandin F_{2alpha} receptor trafficking to early endosome-Rab5a. Panel A shows representative micrographs of the colocalization of FP receptor with Rab5a obtained from cells treated with BSA (A-F) or 0.03% fish oil (G-L), following $PGF_{2\alpha}$ treatment. Panel B shows mean colocalization of FP receptor with Rab5a for luteal cells obtained from cells treated with BSA (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar). *Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. Micron bar represents 20 µm.

Endosome marker, Rab7, was used to determine if the FP receptor was being trafficked to a late endosome for degradation. There was an increase in percent colocalization of Rab7 with the FP receptor for control cells at 60 and 120 min post-$PGF_{2\alpha}$ when compared to 0 min ($P < 0.05$; FIG 23A and FIG 23B; Appendix L). There was no difference in percent colocalization of Rab7 with the FP receptor for fish oil-treated cells following the addition of $PGF_{2\alpha}$ at any time points ($P > 0.05$; FIG 23A and FIG 23B; Appendix M).

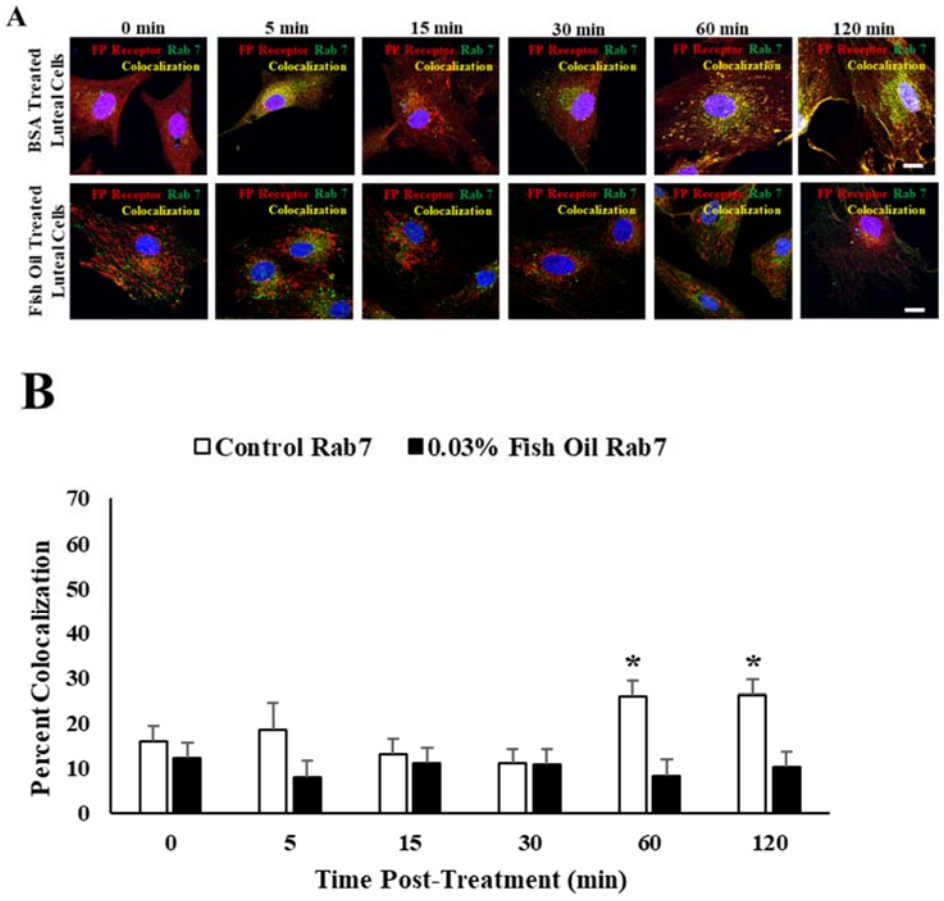

Figure 23. Effects of fish oil on prostaglandin F_{2alpha} receptor trafficking to late endosome- Rab7. Panel A shows representative micrographs of the colocalization of prostaglandin $F_{2\alpha}$ (FP) receptor with Rab7 obtained from cells treated with BSA (A-F), or 0.03% (vol/vol) fish oil (G-L) following treatment with prostaglandin (PG) $F_{2\alpha}$. Panel B shows mean colocalization of FP receptor with Rab7 for luteal cells obtained from BSA control (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar). * Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. Micron bar represents 20 μm.

Recycling endosome marker, Rab11a, was used to determine if the FP receptor was being recycled back to the plasma membrane following $PGF_{2\alpha}$-induced internalization. There was an increase percent colocalization for Rab11a with the FP receptor for control cells at 30 and 120 min post-$PGF_{2\alpha}$ when compared to 0 min ($P < 0.05$; FIG 24A and FIG 24B; Appendix N). However, there was no difference in percent

colocalization for cells treated with fish oil following the addition of $PGF_{2\alpha}$ at any time points (P > 0.05; FIG 24A and FIG 24B; Appendix O).

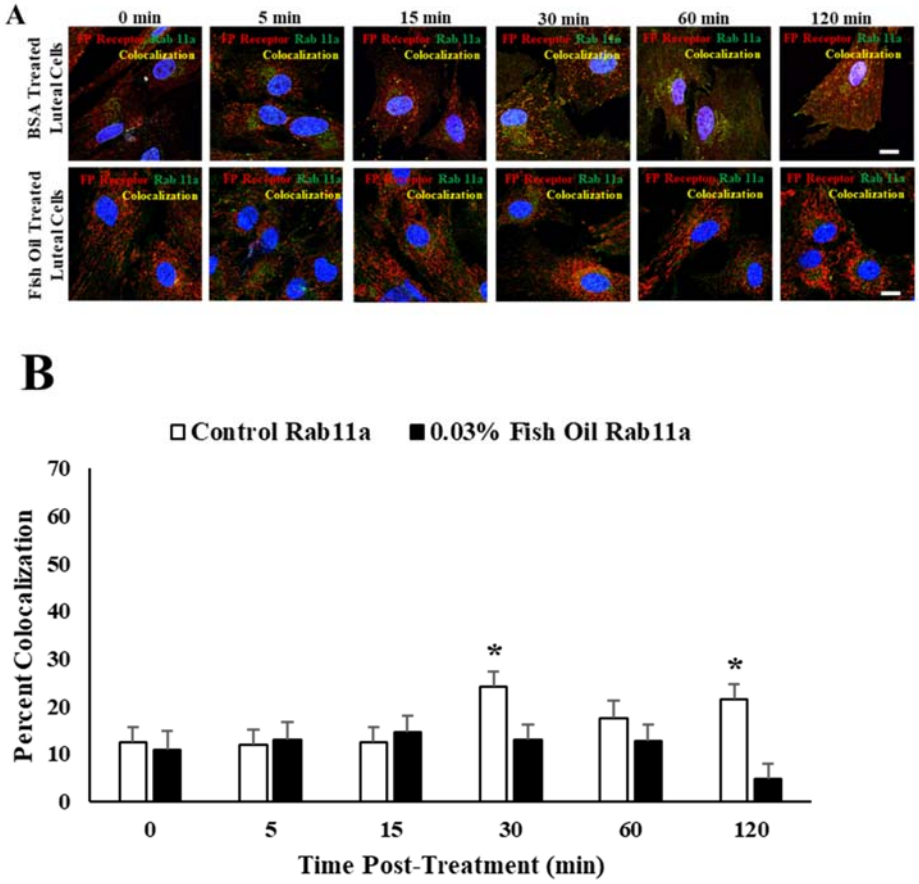

Figure 24. Effects of fish oil on prostaglandin F_{2alpha} receptor trafficking to recycling endosome-Rab11a. Panel A shows representative micrographs of the colocalization of FP receptor with Rab11a obtained from cells treated with BSA (A-F) or 0.03% fish oil (G-L), following $PGF_{2\alpha}$ treatment. Panel B shows mean colocalization of FP receptor with Rab11a for luteal cells obtained from cells treated with BSA (n = 6 CL; open bar) or 0.03% fish oil (n = 6 CL; solid bar). * Significant difference within media supplementation (BSA or fish oil), $P < 0.05$. Micron bar represents 20 µm.

Effects of Fish Oil on Cytoskeleton Integrity in Bovine Luteal Cells

Morphological changes such as punctation or aggregated within actin and tubulin filaments or irregular distribution within the cells are indicators of cytoskeleton disruption (117, 118). There were no obvious morphological differences in both actin or tubulin structure between treatment groups (FIG 25).

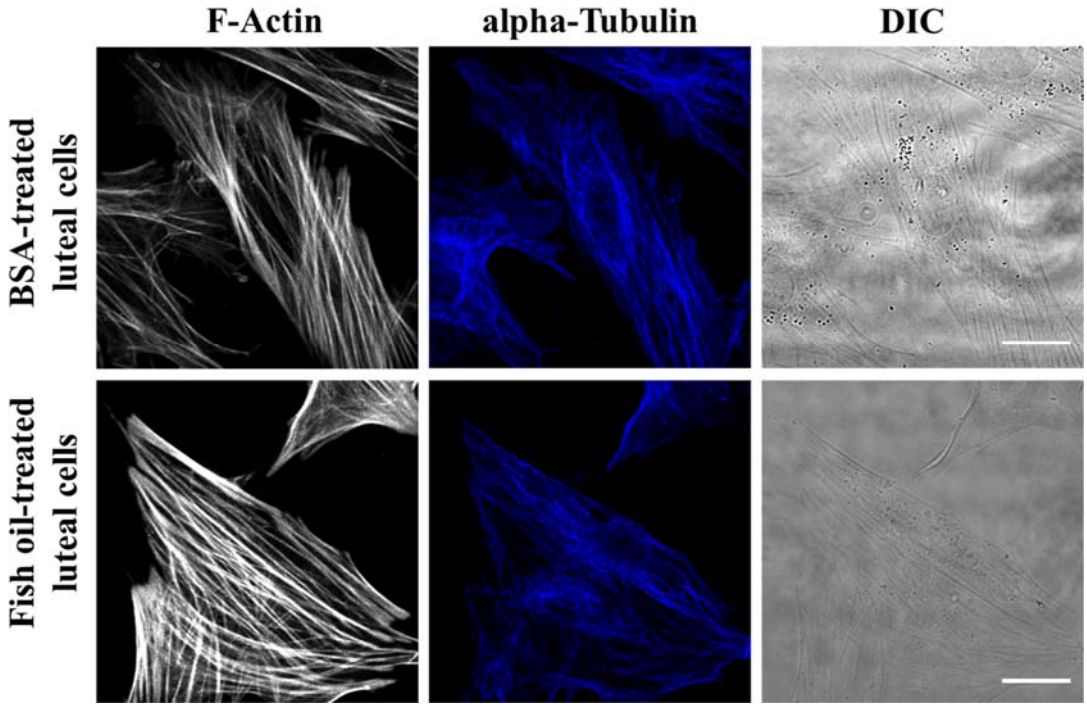

Figure 25. The effects of fish oil on cytoskeleton integrity in bovine luteal cells. Representative micrographs obtained from luteal cells supplemented with BSA or 0.03% (vol/vol) fish oil. From left to right represent F-actin, alpha-tubulin, and differential interference contrast (DIC). Micron bar represents 20 μm.

Effects of Fish Oil on Prostaglandin $F_{2\alpha}$ Induced Mitogen-Activated Protein Kinase Signaling in Bovine Luteal Cells

Mitogen-activated protein kinase signaling regulates the activities of several transcription factors, some of which are critical for regulation of cell cycle (119, 120) and apoptosis (121, 122). There was a 3.2-fold increase at 15 min of phosphorylated MAPK 1/3 following $PGF_{2\alpha}$ treatment for control cells when compared to saline-treated control cells ($P < 0.05$; FIG 26B). Fish oil had no influence on fold induction of phosphorylated MAPK 1/3 following $PGF_{2\alpha}$ treatment when compared to fish oil cells treated with medium alone. There was however, a 1.6-fold decrease at 15 min of phosphorylated MAPK 1/3 following $PGF_{2\alpha}$ treatment for fish oil-treated cells when compared to $PGF_{2\alpha}$-treated control cells ($P < 0.05$; FIG 26B). Additionally, there was a 1.8 and 1.5-fold increase at 15 min of MAPK 1/3 following PDBu treatment control and fish oil-treated cells when compared to control cells, respectively ($P < 0.05$; FIG 26B). This increase fold induction of MAPK 1/3 following PDBu treatment did not differ between control and fish oil-treated cells ($P > 0.05$).

There was a 1.7-fold at 15 min increase of MAPK 14 following $PGF_{2\alpha}$ treatment for control cells when compared to control cells ($P < 0.05$; FIG 26C). Fish oil had no influence on fold induction of MAPK 14 following $PGF_{2\alpha}$ treatment when compared to saline-treated fish oil cells. There was however, a 2.5-fold decrease at 15 min of MAPK 14 following $PGF_{2\alpha}$ treatment for fish oil-treated cells when compared to $PGF_{2\alpha}$-treated control cells ($P < 0.05$; FIG 26C). Additionally, there was a 2.5 and 2-fold increase at 15 min of MAPK 14 following PDBu treatment control and fish oil-treated cells when compared to control cells, respectively ($P < 0.05$; FIG 26C). This increase in fold

induction of MAPK 14 following PDBu treatment did not differ between control and fish oil-treated cells ($P > 0.05$; FIG 26C).

There was a 6.5-fold increase at 15 min of MAPK 8/9 following $PGF_{2\alpha}$ treatment for control cells when compared to saline-treated control cells ($P < 0.05$; FIG 26D). Fish oil had no influence on fold induction of MAPK 8/9 following $PGF_{2\alpha}$ treatment when compared to saline-treated fish oil cells. There was, however, a 9-fold decrease at 15 min of MAPK 8/9 following $PGF_{2\alpha}$ treatment for fish oil-treated cells when compared to $PGF_{2\alpha}$-treated control cells ($P < 0.05$; FIG 26D). Additionally, as seen for both MAPK 1/3 and MAPK 14, there was a 2.2 and 1.8-fold increase at 15 min of MAPK 8/9 following PDBu treatment control and fish oil-treated cells when compared to control cells, respectively ($P < 0.05$; FIG 26D); however, this increase in fold induction of MAPK 8/9 following PDBu treatment did not differ between control and fish oil-treated cells ($P > 0.05$).

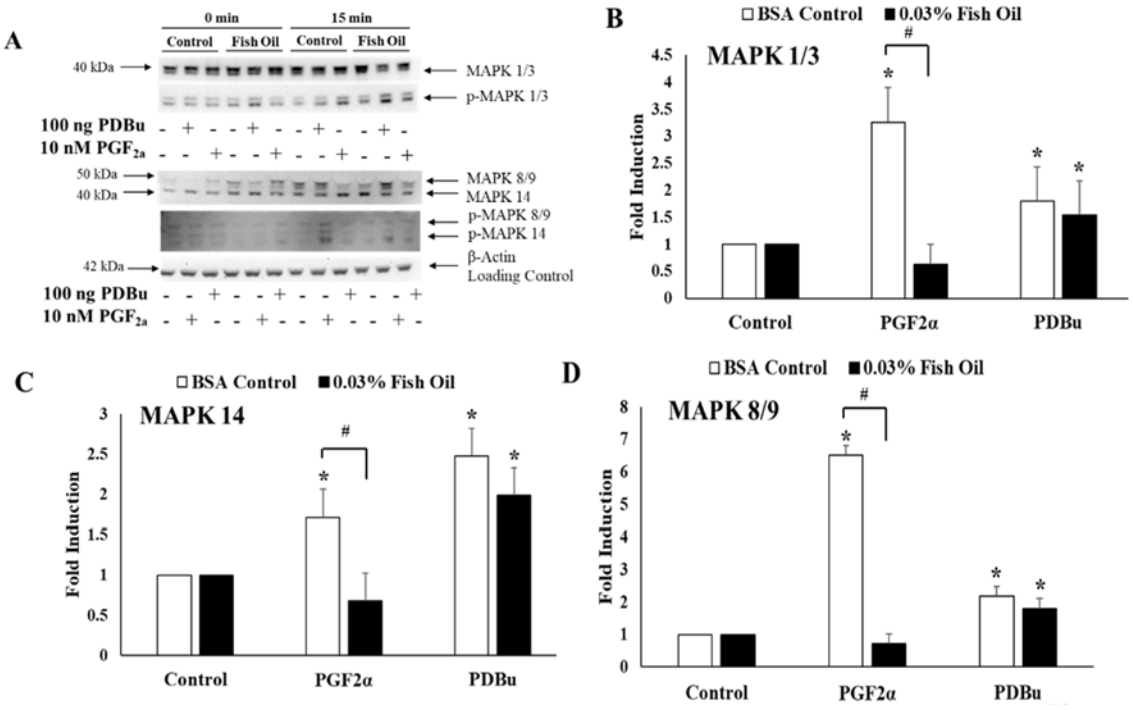

Figure 26. Effects of fish oil on prostaglandin F_{2alpha} induced mitogen-activated protein kinase signaling in bovine luteal cells. Panel A: Representative western blot analysis showing the effects of fish oil on prostaglandin (PG) $F_{2\alpha}$-induced mitogen-activated protein kinase (MAPK) signaling in bovine luteal cells. Panel B: Quantitative results from western blot analysis showing the effects of fish oil on $PGF_{2\alpha}$-induced MAPK 1/3 expression. BSA control (n = 4 CL; open bar) or 0.03% (vol/vol) fish oil (n = 4 CL; solid bar). Panel C: Quantitative results from western blot analysis showing the effects of fish oil on $PGF_{2\alpha}$-induced MAPK 14 expression. BSA control (n = 4 CL; open bar) or 0.03% fish oil (n = 4 CL; solid bar). Panel D: Quantitative results from western blot analysis showing the effects of fish oil on $PGF_{2\alpha}$-induced MAPK 8/9 expression. BSA control (n = 4 CL; open bar) or 0.03% fish oil (n = 4 CL; solid bar). * Significant difference within media supplementation (BSA or fish oil) as compared to control treatment, $P < 0.05$. # Significant difference within treatment group ($PGF_{2\alpha}$ or phorbol-ester (PDBu)), $P < 0.05$.

Discussion

Recent studies from our laboratory have demonstrated that both fish oil and dietary supplementation of fish meal alters lipid microdomain structure (M.R Plewes, et al. unpublished) and affects mobility of the FP receptor in bovine luteal cells (70, 116). However, these studies have yet to couple membrane mobility with downstream

signaling. Here, we demonstrate that fish oil has a major influence on the FP receptor signaling and receptor internalization.

The FP receptor is a GPCR found on the plasma membrane of bovine luteal and endothelial cells (123-125). It has been well-documented that the FP receptor signals through $G_{\alpha q}$, leading to activation of MAPK signaling, resulting in apoptosis (121, 122, 126). Additionally, $G_{\alpha q}$ subunit has been shown to segregate in the caveolae microdomains (67), which may be influenced by incorporation of omega-3 polyunsaturated fatty acids. Methodologies used in the study of lipid microdomains, specifically caveolae, and interactions with trimeric G-proteins are often done by disrupting cell membranes with detergents or mechanically with ultrasonification, and isolation using sucrose density gradient centrifugation with visualization using western blotting (63, 127, 128). While this approach is successful for studying membrane protein and lipid interactions within lipid microdomains, it limits the ability to study the different interactions between proteins and individual linear or caveolae-domains. To our knowledge, this is the first study to examine the association between caveolae and $G_{\alpha q}$, without disrupting the natural aggregation of lipid microdomains in bovine luteal cells.

Fish oil and dietary supplementation of fish meal has been reported to disrupt the lipid-protein interactions of caveolin-1, a critical structural protein in caveolae domains, and the FP receptor in bovine luteal cells (M.R Plewes, et al. unpublished). Results from the current study build on previous work, showing that fish oil not only disrupts lipid-protein interactions of the FP receptor, but also disrupts interactions with $G_{\alpha q}$. It is well-documented that disruption of lipid microdomains often disrupts G-protein localization, attenuating signal transduction (129, 130). Fish oil appeared to have the same influence

on $G_{\alpha q}$ displacement, leading to attenuated $PGF_{2\alpha}$-induced MAPK signaling. However, MAPK signaling was recovered when protein kinase C was activated downstream of the FP receptor. Activation of MAPK signaling using an intracellular-agonist such as phorbol ester, indicates that fish oil affects $PGF_{2\alpha}$ signaling at the level of the plasma membrane, most likely interfering with receptor dynamics and activity. While the current study clearly indicates fish oil disrupts both $G_{\alpha q}$ localization with the FP receptor and $PGF_{2\alpha}$-induced MAPK signaling, the mechanism is still unknown. It is possible that fish oil disrupts the structural conformation of the receptor, preventing ligand binding; however, it is most likely occurring through disruption of protein-protein interactions between the FP receptor and the G-protein alpha subunit. Further work is necessary to determine the mechanism by which fish oil disrupts $PGF_{2\alpha}$-induced MAPK signaling.

Once a signaling cascade is activated, receptor internalization is critical for desensitization, thereby controlling intracellular signaling and response (79, 84, 104). Many GPCRs utilize clathrin-dependent endocytosis for receptor internalization and further trafficking to early endosomes (81, 85). Here we show for the first-time receptor internalization and endosomal trafficking of the FP receptor in bovine luteal cells. In the present study, following clathrin-mediated receptor internalization, the FP receptor was trafficked to early endosomes where some of the FP receptors were further sorted through recycling endosome. Additionally, many FP receptors were trafficked to late endosomes, where the fate would be degradation and desensitization of $PGF_{2\alpha}$ signaling. Additionally, fish oil had no influence on $PGF_{2\alpha}$-induced clathrin-mediated receptor internalization. However, following internalization with clathrin, the FP receptor appeared to be trapped, possibly at the level of the plasma membrane. Fish oil also

appeared to inhibit clathrin-mediated endosomal protein trafficking, both with early endosomes, as well at late and recycling endosomes.

Cytoskeleton plays a critical role in clathrin-mediated protein trafficking to endosomes (131). Omega-3 polyunsaturated fatty acids have been reported to impede key regulatory genes involved in cytoskeleton structural dynamic (86), which may disrupt regulation of protein trafficking. Morphological changes such as punctation within filaments or aggregated actin distributed irregularly within the cells are indicators of cytoskeleton disruption (117, 118). In the current study, fish oil had no influence on gross morphology of actin or tubulin filaments. However, the methodology used to determine the effects of fish oil on actin structural integrity was restricted to gross morphology. Therefore, it is unknown whether disruption of endosomal trafficking is due to disorder with adaptor proteins involved with the assembly of clathrin pits or whether fish oil effects cytoskeleton integrity, which was undetected through microscopy. Nonetheless, fish oil significantly effects internalization and trafficking of the FP receptor, which may allow for the generation of novel mechanistic approaches for attenuating the luteolytic effects of $PGF_{2\alpha}$.

Regulation of uterine $PGF_{2\alpha}$ secretion during the period of maternal recognition of pregnancy is critical for successful pregnancy (132, 133). Inclusion of fish meal in the diet of dairy and beef cows has been reported to improve reproductive performance (43, 134, 135). Most studies have focused on the effects of fish meal on uterine $PGF_{2\alpha}$ secretion. However, fish meal influences CL function as well. Previous results from our laboratory demonstrated supplementation of fish meal luteal sensitivity to $PGF_{2\alpha}$ (114). Results from the present study provide insight into the mechanism by which fish meal

decreases luteal sensitivity to $PGF_{2\alpha}$. Here, we demonstrate that fish oil disrupts the colocalization of $G_{\alpha q}$ with both caveolae microdomains as well as colocalization with the FP receptor. This disruption of the FP receptor with the G-protein alpha subunit may be a mechanism by which MAPK signaling is diminished following the addition of $PGF_{2\alpha}$. In addition to disrupting colocalization of $G_{\alpha q}$ with both caveolae and the FP receptor, fish oil disrupts FP receptor internalization and endosomal protein trafficking. Taken together, inclusion of fish oil or meal in the diet of breeding females may be a novel approach for attenuating $PGF_{2\alpha}$ signaling, and possibly increasing the window for the developing embryo to rescue the CL.

CHAPTER 4

INFLUENCE OF OMEGA-3 FATTY ACIDS ON BOVINE LUTEAL CELL PLASMA MEMBRANE DYNAMICS

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Contribution of Authors and Co-Authors

Manuscript in Chapter IV

Author: Michele R. Plewes

Contributions: Developed and implemented the study design. Generated and analyzed data. Wrote first draft of the manuscript.

Co-Author: Patrick. D. Burns

Contributions: Helped conceive the study topic. Provided guidance on study design. Provided feedback data interpretation and manuscript preparation.

Co-Author: Richard M. Hyslop

Contributions: Helped conceive the study topic. Provided feedback data interpretation and manuscript preparation.

Co-Author: B. George Barisas

Contributions: Helped conceive the study topic. Provided guidance on study design. Provided feedback data interpretation and manuscript preparation.

Abstract

Fish oil is a rich source of omega-3 fatty acids which disrupt lipid microdomain structure and affect mobility of the prostaglandin $F_{2\alpha}$ (FP) receptor in bovine luteal cells. The objectives of this study were to determine the effects of individual omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on 1) membrane fatty acid composition, 2) lipid microdomain structure, and 3) lateral mobility of the FP receptor in bovine luteal cells. Ovaries were collected from a local abattoir (n = 5/experiment). The corpus luteum was resected and enzymatically digested using collagenase to generate a mixed luteal cell population. In all experiments, luteal cells were treated with 0, 1, 10 or 100 μM EPA or DHA for 72 h to allow incorporation of fatty acids into membrane lipids. Results from experiment 1 show that culturing luteal cells in the presence of EPA or DHA increased these luteal fatty acids in a dose-dependent manner. In experiment 2, both EPA and DHA increased spatial distribution of lipid microdomains in a dose-dependent manner. Single particle tracking results from experiment 3 show that increasing both EPA and DHA concentrations increased micro- and macro-diffusion coefficients, increased domain size, and decreased residence time of FP receptors. Collectively, results from this study demonstrate similar effects of EPA and DHA on lipid microdomain structure and lateral mobility of FP receptors in cultured bovine luteal cells.

Introduction

The corpus luteum (CL) is a transient endocrine gland formed from the ovulatory follicle following ovulation. Progesterone is a steroid hormone synthesized and secreted by the gland that is vital for the establishment and maintenance of pregnancy in the cow

(8). In the absence of an embryo, the uterus releases prostaglandin (PG) $F_{2\alpha}$ in a series of 6 - 8 pulses at approximately days 15 to 18 following ovulation (136, 137). Luteal cells of the CL express FP receptors (138, 139), a G-protein coupled, membrane-bound receptor, and binding of $PGF_{2\alpha}$ triggers an intracellular signaling cascade that causes regression of the gland (140, 141), allowing for another opportunity to become pregnant. In the pregnant cow, the embryo must inhibit uterine $PGF_{2\alpha}$ secretion to maintain the structure and function of the CL. Embryonic mortality often occurs when a viable embryo fails to effectively control maternal $PGF_{2\alpha}$ secretion, which results in the regression of the CL and termination of the pregnancy (10, 142, 143). Therefore, diminishing or altering sensitivity of luteal cells to $PGF_{2\alpha}$ during early pregnancy may prevent regression of the CL.

The plasma membrane of cells is a complex and highly dynamic structure composed of lipids, carbohydrates, and proteins (144, 145). The lipids form a bilayer that serves as a selective barrier separating the external from the internal environment of the cell. There are three major lipid classes found within mammalian biological membranes, namely glycerophospholipids, cholesterol, and sphingolipids. Glycerophospholipids are the predominate lipid of the plasma membrane that forms the lipid bilayer. Cholesterol tightly packs and interacts with sphingolipids forming distinct patches 10 to 200 nm in diameter referred to as lipid microdomains (61). The interaction between cholesterol and sphingolipids limits incorporation into the glycerophospholipid bilayer, causing it to form detergent-resistant microdomains (146). This unique environment between cholesterol and sphingolipids plays a role in recruitment of membrane-bound receptors and associated intracellular signaling molecules allowing for downstream signal transduction

within the cell (96, 147, 148). Therefore, altering lipid microdomain structure of luteal cells may reduce sensitivity to the luteolytic actions of $PGF_{2\alpha}$.

Long-chain saturated, unsaturated, and polyunsaturated fatty acids are the basic constituents of glycerophospholipids and sphingolipids found within the plasma membrane. These fatty acids play a vital role in the structural integrity of the plasma membrane, membrane fluidity, and signal transduction. Omega-3 fatty acids are a class of long-chain polyunsaturated fatty acids with the first double bond at the third carbon atom from the omega terminal end. Alpha-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are common omega-3 fatty acids found within biological membranes, including the plasma membrane. The high degree of unsaturation of EPA and DHA, five and six double bonds, respectively, can hinder the quasi liquid-order of lipid microdomains (149). Therefore, increasing either EPA or DHA into glycerophospholipids and sphingolipids may influence microdomain structure.

Fish oil obtained from cold water fishes is abundant in both EPA and DHA, and supplementing the diet with fish oil or fish meal has been reported to affect lipid microdomain structure (53, 70, 74, 96, 97). Furthermore, a recent study from this laboratory demonstrated increased lateral mobility of the FP receptor in bovine luteal cells treated with fish oil (70). However, which omega-3 fatty acid in fish oil affects lipid microdomain structure is largely unknown. Most studies show that DHA may have greater influence on the disruption of lipid microdomains (54, 87, 96, 150, 151), despite the fact that EPA alters microdomain structure and lipid composition as well (149). The objectives of this study were to determine the effects of fish oil, EPA, and DHA on 1) luteal cell fatty acid composition, 2) lipid microdomain structure, and 3) lateral mobility

of the FP receptor on the plasma membrane of bovine luteal cells. Understanding the influence that individual omega-3 fatty acids, EPA and DHA, have on membrane structure is critical for making the appropriate dietary recommendations for improving reproduction in mammalian females.

Methods

Tissue Collection, Cell Preparation, and Cell Culture

Bovine ovaries containing a CL were collected at a local abattoir and transported to the laboratory at the University of Northern Colorado in 1× sterile PBS. Gross ovarian morphology was used to determine the age of the CL as previously described (152) and only mature CL (n = 5/experiment) were used in these studies. Ovaries with a mature CL were then submerged in 70% ethanol and 0.06% quaternary ammonium to destroy any microorganisms that may be present on the outside of the ovary from time of collection.

The CL was removed from the ovary using sterile techniques under a laminar flow hood, and placed into a sterile 60-mm² Petri dish containing 10 mL of ice-cold Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS, pH 7.34). Capsular connective tissue was carefully dissected away to expose luteal tissue. Luteal tissue was removed and cut into approximately 1 mm^3 fragments and 1 g of tissue was placed into T-25 plugged culture flasks containing 5 mL dissociation medium (HBSS containing 2000 units of collagenase type 1 per g tissue and 0.1% BSA). Tissue was incubated at 37 °C for 45 min in a shaking water bath. Following incubation, the supernatant was removed and transferred to a sterile 15-mL culture tube. Cells were then washed 3 × with sterile 1× PBS and re-suspended in 10 mL of culture medium (Ham's F12 supplemented with 5% fetal bovine serum, insulin-transferrin-selenium-supplement (Invitrogen, Carlsbad, CA,

USA), 1× antibiotic-antimycotic (Gibco, Waltham, Massachusetts, USA), pH 7.34), until cell concentration and viability was determined.

Trypan blue was used to determine the viability of cells, and a hemocytometer was used to estimate cell concentration. In the current study, only preparations with a cell population of greater than 85% viability were used for each experiment. Cell cultures were maintained at 37 °C in an atmosphere of 95% humidified air and 5% CO_2.

Lipid Preparation for In Vitro Culture

Eicosapentaenoic acid and DHA were purchased form Cayman Chemical (Ann Arbor, MI, USA) and added to culture medium containing 33 mg/mL fatty acid-free BSA at the appropriate concentration for each experiment as described by Mattos et al. (88). Fatty acids from a commercial fish oil (Pharmavite, Mission Hills, CA) were also pre-bound to BSA prior to the addition to culture as previously described (70). Control medium was prepared as stated above without the addition of lipids.

Experiment 1: Effects of Fish Oil, Eicosapentaenoic, and Docosahexaenoic Acids on Omega-3 Polyunsaturated Fatty Acid Composition of Bovine Luteal Cells

Mixed luteal cells were plated in T-75 culture flasks at 2×10^6 cells/flask (n = 2 T-75 flasks/treatment/CL). Cells were maintained at 37 °C in an atmosphere of 95% humidified air and 5% CO_2 until flasks reached 85 to 90% confluency. After reaching desired confluency, culture medium was removed and cells were treated with control medium, fish oil (0.3% vol/vol), or medium supplemented with EPA or DHA at 1, 10 or

100 μM. Cells were cultured an additional 72 h with supplemented medium to allow for incorporation of long-chain fatty acids into biological membranes. Following incubation, cells were removed from culture flasks and washed 6 × with 1× sterile PBS to remove excess free fatty acids. Cells were placed into a 16 × 100 mm reaction tube and freeze-dried for 24 h prior to methylation. Samples were maintained in the dark to minimize light-induced oxidation of fatty acids. Fatty acids were methylated using direct methylation as previously described (72).

An Agilent 7890A Series gas-liquid chromatograph (Wilmington, DE, USA) with a mass spectrometer (GC-MS) detector was used to determine long-chain fatty acid composition. The instrument was equipped with a 30-m × 0.20-mm (i.d.) fused silica capillary column (Supelcowax10; Supelco Inc., Bellefonte, PA, USA) and electron impact ionization was used. The injector temperature was set at 250 °C and 1 μL of fatty acid methyl ester samples was applied to the column using split-less mode. The carrier gas utilized was helium with a flow rate of 1 mL/min. Oven temperature was programmed from an initial temperature of 140 °C, which was held for 10 min, and then increased to a final temperature of 250 °C at the rate of 2.5 °C/min. The final temperature of 250 °C was held constant for 10 min for a total run time of 65 min. Standard fatty acid methyl ester mixtures were used to calibrate the instrument using reference standard GLC 68-D (Nu-Chek Prep, Inc. Waterville, MN, USA). Chromatograms were generated for each analysis using ChemStation Plus Chromatograph Manager (Agilent Technologies, Boulder, CO, USA). Identification of long-chain fatty acids obtained from cultured cells were determined by comparing the mass spectrometry analysis and relative retention

times of fatty acid to the known set of standards. These peaks were then calculated as normalized area percentages of fatty acids.

Experiment 2: Effects of Fish Oil, Eicosapentaenoic, and Docosahexaenoic Acids on Spatial Distribution of Lipid Microdomains

Mixed luteal cells were plated in four-chamber glass-bottom culture dishes (Cellvis, Mountain View, CA, USA) at 5×10^4 cells/dish (n = 2 chambers/treatment). Cells were incubated overnight at 37 °C in an atmosphere of 95% humidified air and 5% CO_2 to allow cells to adhere to glass cover slips. Culture medium was removed and cells were treated with supplemented medium as described in experiment 1 for 72 h allowing for incorporation of fatty acids into cell membranes.

Lipid microdomain labeling and visualization. Lipid microdomains were labeled as previously described and validated in our laboratory (70, 153). Following labeling of microdomains, cells were viewed using a Zeiss confocal microscope using a 40× water immersion objective (1.2 N.A). The appropriate laser was used to excite the Alexa-555 fluorophore. Approximately 10-15 cells were randomly selected from each dish and 1 μm slice images were generated from bottom to top of each cell. Whole cell fluorescence intensity was determined from three-dimensional images using ImageJ software. A total of 55, 69, 42, 55, 48, 32, 45, and 74 mixed luteal cells were analyzed from control, 0.03% fish oil (vol/vol), 1, 10, 100 μM EPA and DHA treatments, respectively.

Experiment 3: Effects of Fish oil, Eicosapentaenoic, and Docosahexaenoic Acids on Lateral Mobility of FP Receptors on the Plasma Membrane of Bovine Luteal Cells

Mixed luteal cells were cultured in four-chamber glass bottom culture dishes at 5 × 10^4 cells/dish. Cells were cultured as described in experiment 2 for 72 h. Cells were then prepared using a validated protocol for single particle tracking as previously described (70). In brief, polyclonal FP receptor antibody (Cayman Chemical, Ann Arbor, MI, USA) was conjugated to biotin per manufacturer's instructions using a DSB-X biotin protein labeling kit (Life Technologies, Carlsbad, CA, USA). Receptors were prepared for single particle tracking experiments using biotinylated FP receptor antibody and Quantum dot® 605 Streptavidin Conjugate (Thermo-Fisher Scientific, Waltham MA, USA).

A Zeiss confocal microscope equipped with a high-speed camera (Hamamatsu Photonics, Japan) was used to capture individual receptor trajectories. Ultraviolet light was used to excite Quantum dots and emission of light was collected as 605 nm. Receptors were visualized using 100× oil objective and an acquisition image size of 512 × 512 pixel (33.3 μm × 33.3 μm). Receptors were recorded for 29 s (30 frames/s) and only receptors containing a minimum of 10 s of recordings were used for the final analysis in this study.

Video Spot Tracker v08.01 software (Computer Integrated Systems for Microcopy and Manipulation, University of North Carolina-Chapel Hill, Chapel Hill, NC, USA) was used to track the motion of the receptor, generating individual X-Y

coordinates. Trajectories were given as X-Y pixel and converted to μm. Mean square displacement of trajectories was calculated and plotted as a function of time to determine both micro- and macro-diffusion coefficients according to the equations reported by Daumas et al (89). Residence time and domain size were determined from each individual receptor as previously described (70). A total of 22, 22, 22, 20, 28, 22, 24, and 25 receptor trajectories were analyzed from control, 0.03% fish oil (vol/vol), 1, 10, 100 μM EPA and DHA supplemented luteal cells, respectively.

Statistical Analysis

All data are reported as least square means ± standard error of the mean and unless otherwise indicated, significance was declared at $P < 0.05$. Effects of EPA and DHA on luteal fatty acid composition, lipid microdomain staining intensity, lateral mobility of FP receptors (micro- and macro- diffusion coefficients), domain sizes, and residence time of receptors were analyzed using one-way analysis of variance (ANOVA). The model included concentration (0, 1, 10, or 100 μM) of fatty acid (EPA or DHA), fish oil, CL, concentration × fatty acid interaction, and residual error as sources of variation. Corpus luteum was considered a random variable in the model. Calculations were made using the mixed-model procedure of SAS (SAS Institute Inc. Cary, USA) and pairwise *t*-tests (PDIFF option) to separate means if ANOVA was significant. The effects of concentration of EPA and DHA on luteal fatty acid composition, lipid microdomain staining intensity and lateral mobility of FP receptor were analyzed using linear regression. Calculations were made using the procedure regression of SAS.

Results

Effects of Fish Oil, Eicosapentaenoic, and Docosahexaenoic Acids on Omega-3 Polyunsaturated Fatty Acid Composition of Bovine Luteal Cells

In experiment 1, GCMS was used to determine the effects of fish oil, EPA, and DHA on long-chain fatty acid composition in cultured bovine luteal cells. Fish oil treatment did not affect content of palmitic, palmitoleic, stearic, oleic, linoleic α-linolenic, and arachidonic fatty acids when compared to control cells (Fig 27A). Luteal content of EPA and DHA was greater for cells treated with fish oil as compared to control cells (Fig 28A). Regardless of concentration, there was no effect of culturing luteal cells in the presence of EPA on content of palmitic, palmitoleic, stearic, oleic, linoleic, α-linolenic, or DHA. There was a linear dose-dependent increase in luteal EPA with increasing concentrations of EPA. Furthermore, EPA was greater for cells treated with 10 and 100 μM EPA when compared to control treated cells (Fig 28B). Luteal EPA was less in cells treated with 1 μM EPA as compared to higher doses, but there was no difference between 10 and 100 μM EPA treated cells. Additionally, cells treated with 100 μM EPA had decreased arachidonic acid when compared to control treated cells (Fig 27B).

Similar results were obtained in cells treated with DHA. Regardless of concentration, there was no effect of DHA on luteal fatty acid content of palmitic, palmitoleic, stearic, oleic, linoleic, α-linolenic, arachidonic acids, or EPA (Fig 27C). As with EPA, there was a linear dose-dependent increase in luteal DHA with increasing concentrations of DHA. Similar to effects of EPA on luteal fatty acid composition, DHA

was greater for cells treated with 10 and 100 μM DHA as compared to control treated cells (Fig 28C). As observed with EPA, luteal DHA was less in cells treated with 1 μM DHA as compared to higher doses, but there was no difference between 10 and 100 μM DHA treated cells (Fig 28C). While 100 μM DHA decreased arachidonic acid by 57% when compared to control cells, this was not significant (Fig 27C).

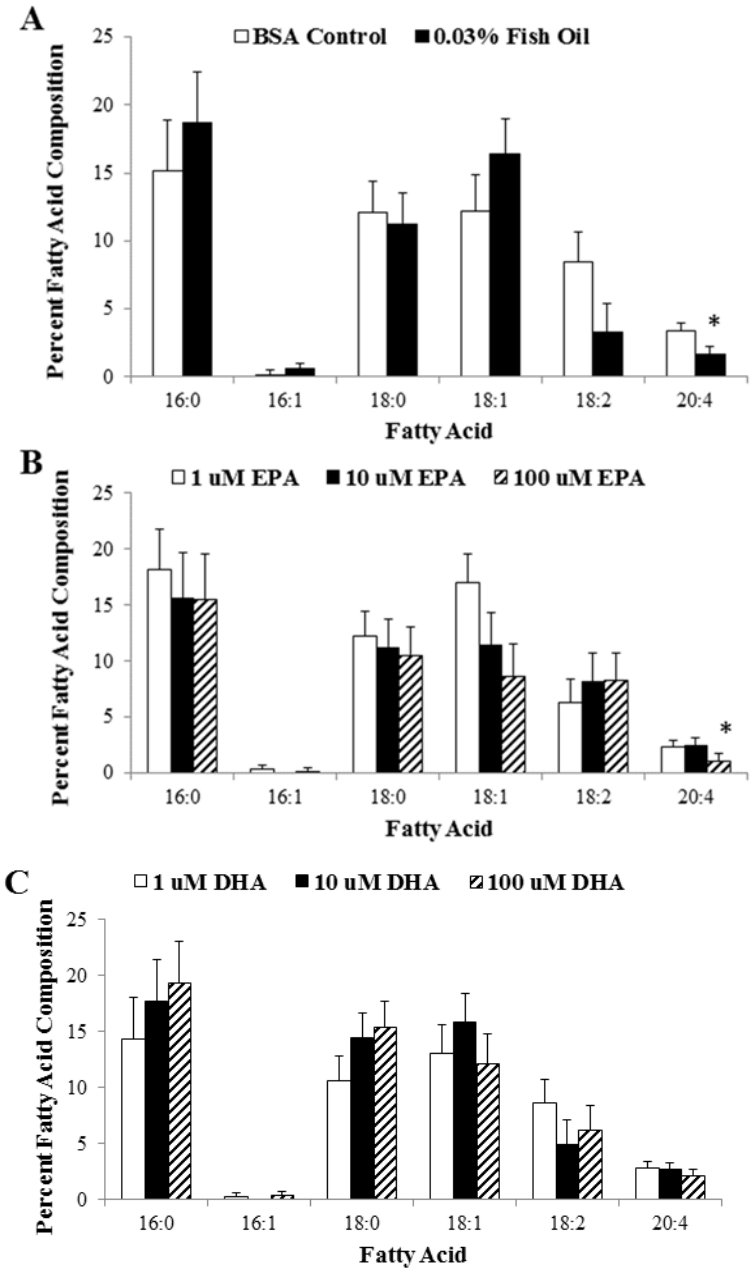

Figure 27. Effects of fish oil, eicosapentaenoic (EPA), and docosahexaenoic acid (DHA) on relative composition of bovine luteal cell long-chain fatty acids. Relative composition (weight percent) of palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linolenic acid (18:2) and arachidonic acid (20:4). Panel A: Bovine serum albumin (BSA) control (n = 5; solid grey bars) and 0.03% fish oil (vol/vol; n = 5; hatched bars). Panel B: 1 µM eicosapentaenoic acid (EPA; n = 5; open bars), 10 µM EPA (n = 5; solid bars), 100 µM EPA (n = 5; hatched bars). Panel C: 1 µM docosahexaenoic acids (DHA; n = 5; open bars) 10 µM DHA (n = 5; solid bars), 100 µM DHA (n = 5; hatched bars). *Significant difference within class of fatty acid compared to BSA control in Panel A; $P < 0.05$.

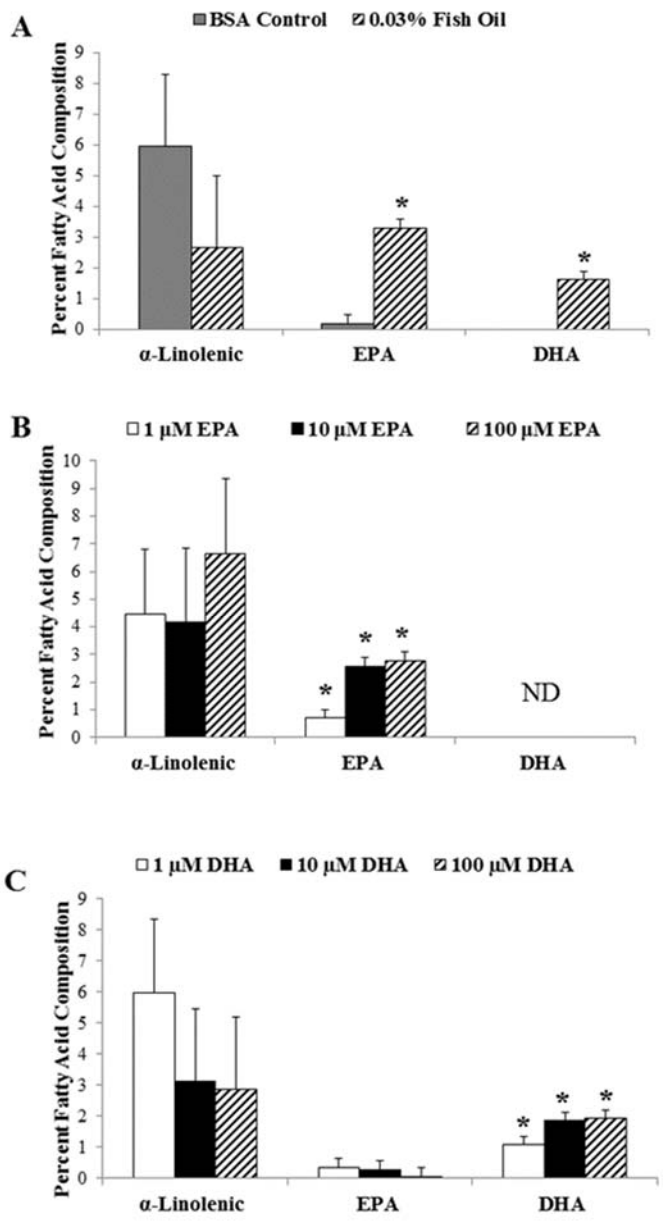

Figure 28. Effects of fish oil, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acid on relative composition (weight percent) of bovine luteal cell omega-3 long-chain polyunsaturated fatty acids. Relative composition (weight percent) of luteal α-linolenic acid (18:3), eicosapentaenoic acid (EPA; 20:5), and docosahexaenoic acid (DHA; 22:6). Panel A: Bovine serum albumin (BSA) control (n = 5; solid grey bars) and 0.03% fish oil (vol/vol; n = 5; hatched bars). Panel B: 1 μM EPA (n = 5; open bars), 10 μM EPA (n = 5; solid bars), 100 μM EPA (n = 5; hatched bars). Panel C: 1 μM DHA (n = 5; open bars), 10 μM DHA (n = 5; solid bars), 100 μM DHA (n = 5; hatched bars). *Significant difference within class of fatty acid compared to BSA control in Panel A; $P < 0.05$. Non-detectable (ND).

Effects of Fish Oil, Eicosapentaenoic, and Docosahexaenoic Acids on Spatial Distribution of Lipid Microdomains

Experiment 2 investigated the effects of fish oil and varying concentrations of EPA and DHA on spatial distribution of lipid microdomains on the plasma membrane of bovine luteal cells. Fish oil decreased total cell fluorescent intensity of microdomains when compared to control treated cells. Increasing both EPA and DHA concentrations from 1 to 100 μM resulted in a linear disruption of lipid microdomains, which was detected by decreased total cell florescent intensity (Fig 29). Both 10 and 100 μM EPA and DHA decreased total cell fluorescent intensity of microdomains when compared to control treated cells, but was not different when compared to fish oil treated cells.

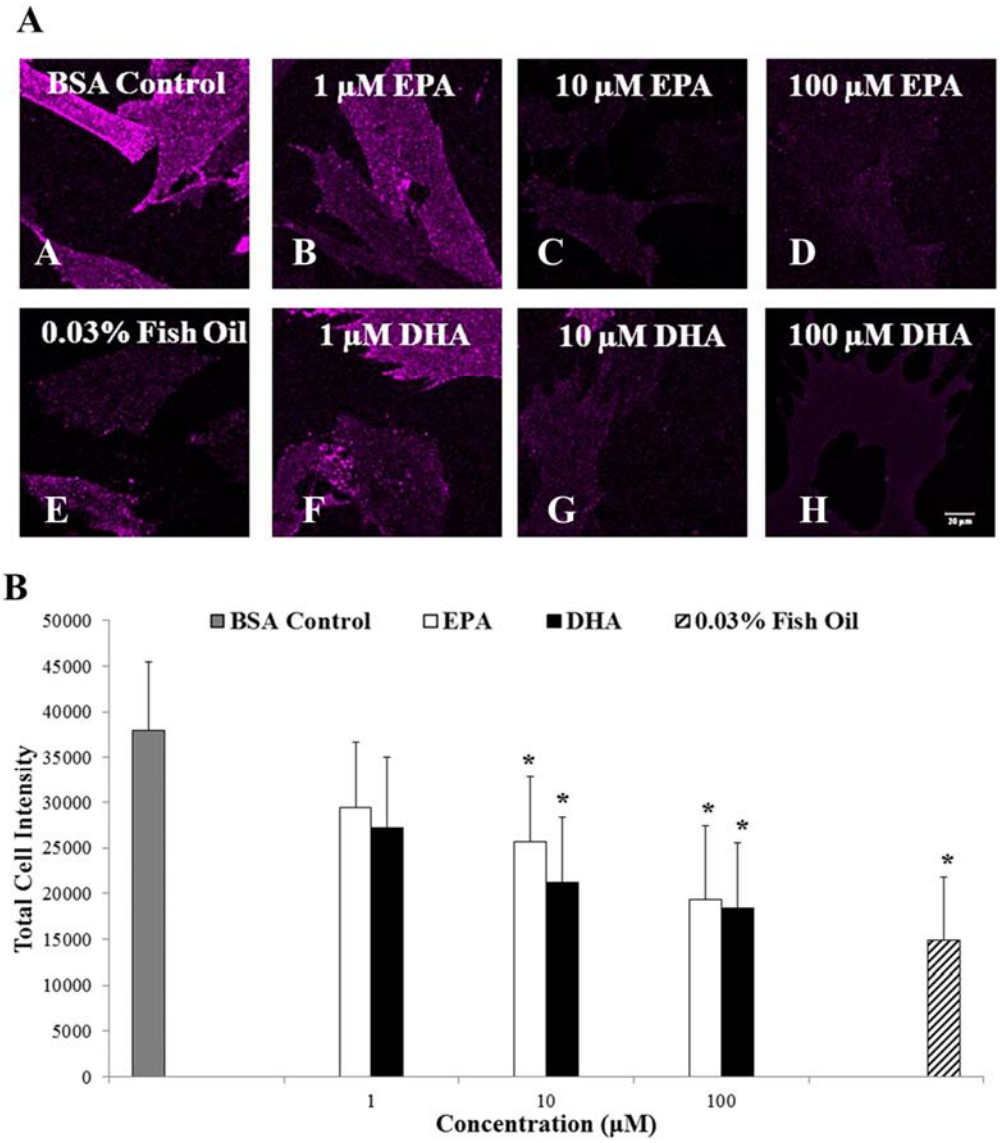

Figure 29. Effects of fish oil, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acid on spatial distribution of lipid microdomains. Panel A: Representative micrographs obtained from (A) Bovine serum albumin (BSA) control, (B) 1 µM EPA, (C) 10 µM EPA, (D) 100 µM EPA, (E) 0.03% fish oil (vol/vol), (F) 1 µM DHA, (G) 10 µM DHA, and (H) 100 µM DHA. Panel B: Mean fluorescent intensity for luteal cells obtained from BSA control, (n = 55 cells), 1 µM EPA (n = 42 cells), 10 µM EPA (n = 55 cells), 100 µM EPA (n = 48 cells), fish oil (n = 69 cells), 1 µM DHA (n = 32 cells), 10 µM DHA (n = 45 cells), and 100 µM DHA (n = 74 cells); BSA control vs. fish oil, EPA or DHA; *$P < 0.05$. Micron bar represents 20 µm.

Effects of Fish Oil, Eicosapentaenoic, and Docosahexaenoic Acids on Lateral Mobility of FP Receptors on the Plasma Membrane of Bovine Luteal Cells

In experiment 3, live-cell single molecule tracking was used to observe lateral mobility of the bovine FP receptors in real time. Eicosapentaenoic acid and DHA had a significant influence on lateral mobility of receptors in a dose dependent manner. There was a linear increase in micro- and macro-diffusion of FP receptors for EPA-treated cells. Similar results were obtained for DHA-treated cells for micro- and macro-diffusion. Both micro- and macro-diffusion of FP receptors were increased in cells treated with 10 and 100 μM EPA and DHA as compared to control cells. Micro-diffusion, or initial velocity of the FP receptor, increased 77 and 126%, for 10 and 100 μM EPA, respectively, when compared to receptors from control cells. Similar results were observed for DHA, with micro-diffusion increasing 105 and 195%, for 10 and 100 μM DHA, respectively, when compared to receptors from control cells (Fig 30A). Additionally, micro-diffusion of FP receptors increased in fish oil treated cells when compared to control cells.

Macro-diffusion, or average velocity of the FP receptor, increased 372 and 426%, for 10 and 100 μM EPA respectively, when compared to receptors from control cells. Similar results were observed for DHA, increasing macro-diffusion by 310 and 351%, for 10 and 100 μM DHA respectively, when compared to receptors from control cells (Fig 30B). Within equivalent concentrations of EPA and DHA, there was no difference in macro-

diffusion. Additionally, macro-diffusion of FP receptors was increased for fish oil treated cells when compared to control cells.

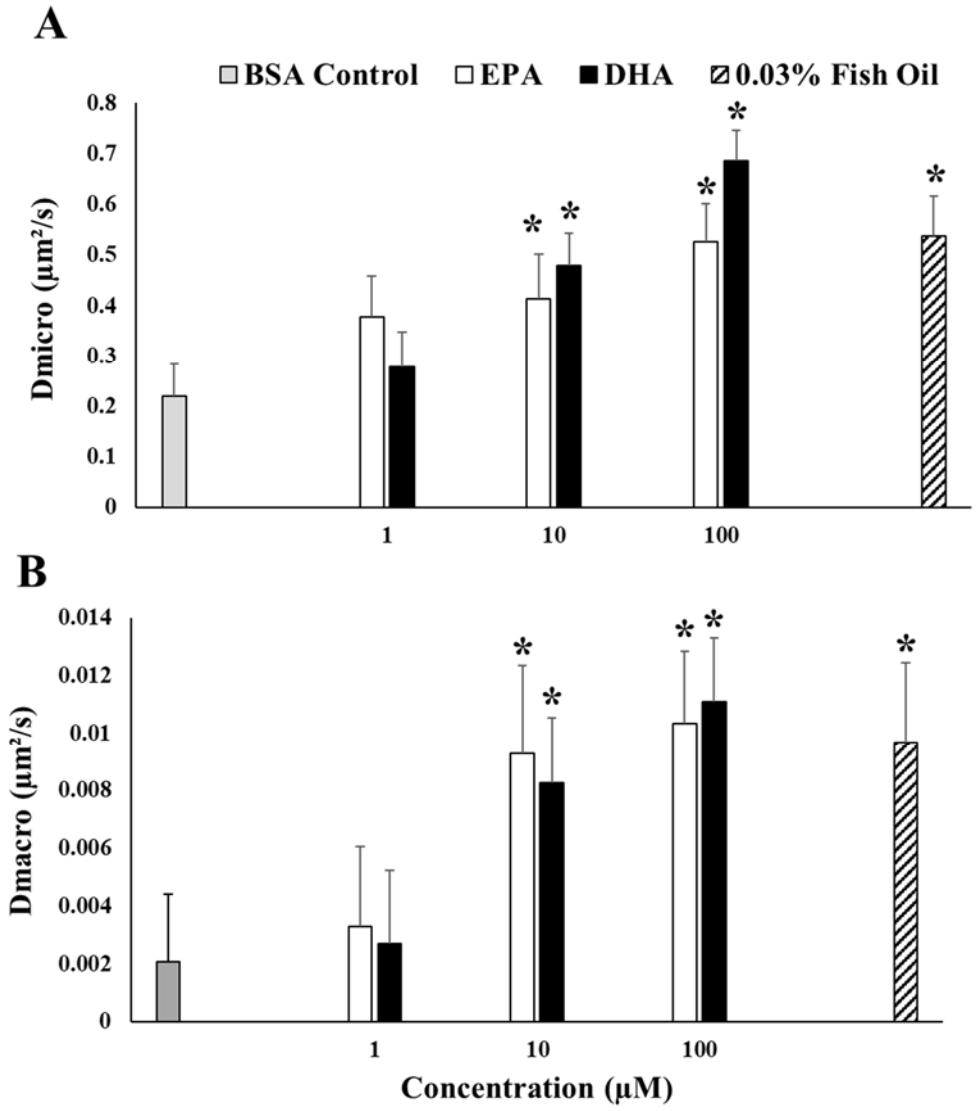

Figure 30. Effects of fish oil, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acid on lateral mobility of prostaglandin F_{2alpha} receptors on bovine luteal cells. (A) micro- and (B) macro-diffusion coefficient of the prostaglandin $F_{2\alpha}$ (FP) receptor on bovine luteal cells treated with Bovine serum albumin (BSA) control medium, (n = 22 FP receptors), 1 µM EPA (n = 22 FP receptors), 1 µM DHA (n = 22 FP receptors), 10 µM EPA (n = 20 FP receptors), 10 µM DHA (n = 24 FP receptors), 100 µM EPA (n = 28 FP receptors), 100 µM DHA (n = 25 FP receptors), and 0.03% fish oil (vol/vol; n = 22 FP receptors); BSA control vs. fish oil, EPA or DHA; mean ± standard error of the mean * $P < 0.05$.

Eicosapentaenoic acid and DHA had a significant influence on domain size and time a FP receptor resided within a domain, which is referred as residence time in a dose dependent manner. There was a linear increase in domain size and decrease in residence time for EPA-treated cells. Similar increase and decrease, respectively, were obtained for DHA-treated cells for domain size and residence time. Both 10 and 100 μM EPA and DHA affected domain size and residence time when compared to control treated-cells. Domain size was increased by 78 and 115% for 10 and 100 μM EPA and by 85 and 116% for 10 and 100 μM DHA, respectively. This is in agreement with spatial distribution data, indicating both EPA and DHA increased disruption of lipid microdomains. Furthermore, domain size was increased in fish oil-treated cells when compared to control cells. The residence time of FP receptors within domains for cells from 10 and 100 μM EPA treated cells decreased by 27 and 34%, respectively, when compared to receptors on control cells (Fig 31B). Residence time of FP receptors was decreased in fish oil-treated cells when compared to control cells. Likewise, the residence time of FP receptors within a domain for cells from 10 and 100 μM DHA treated cells was decreased by 30 and 31%, respectively, when compared to receptors from control cells (Fig 31B).

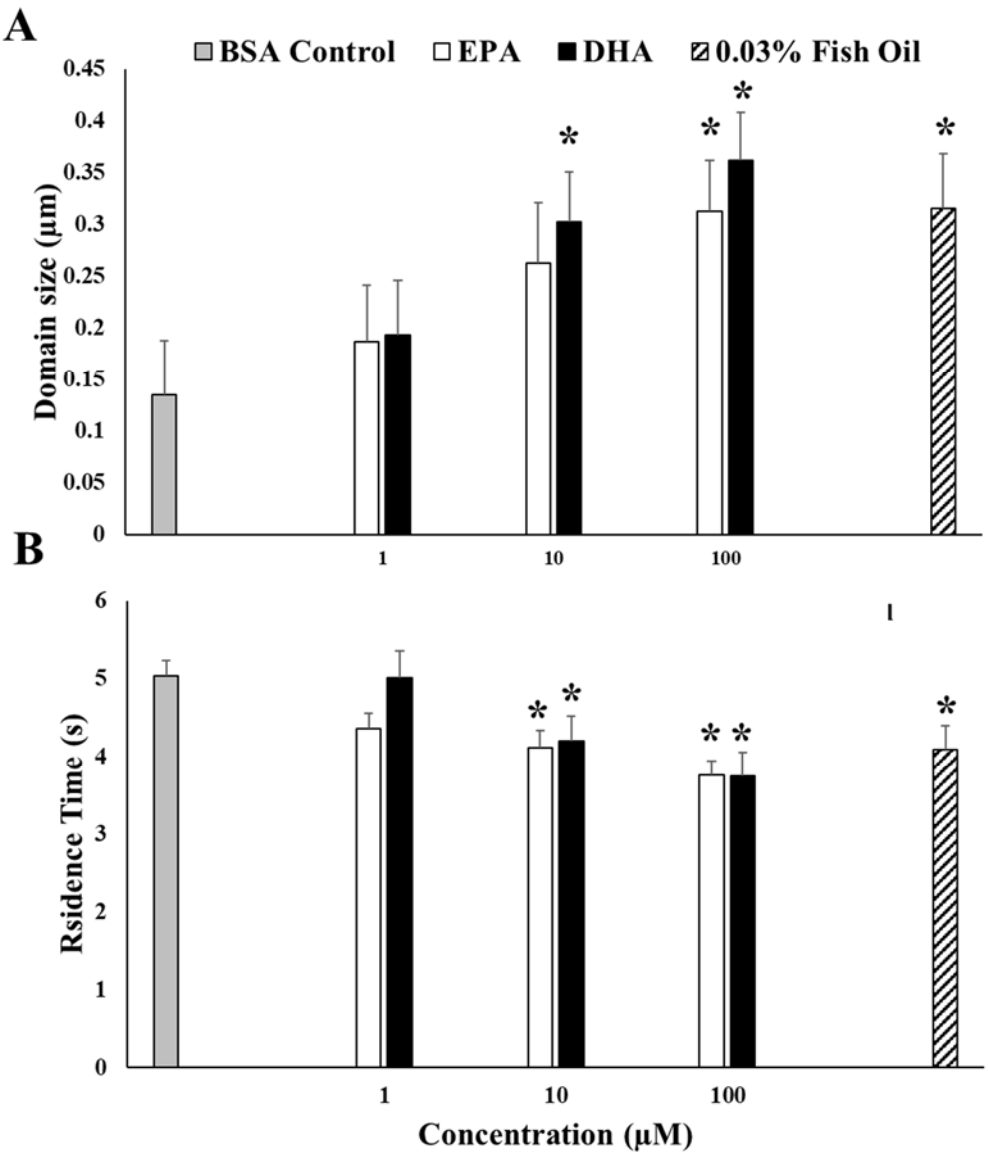

Figure 31. Effects of fish oil, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acid on domain size and residence time of prostaglandin F_{2alpha} receptors on bovine luteal cells. (A) domain size and (B) residence time of the prostaglandin $F_{2\alpha}$ (FP) receptors on bovine luteal cells treated with Bovine serum albumin (BSA) control medium, (n = 22 FP receptors), 1 µM EPA (n = 22 FP receptors), 1 µM DHA (n = 22 FP receptors), 10 µM EPA (n = 18 FP receptors), 10 µM DHA (n = 24 FP receptors), 100 µM EPA (n = 28 FP receptors), 100 µM DHA (n = 25 FP receptors), and 0.03% fish oil (vol/vol; n = 22 FP receptors); BSA control vs. fish oil, EPA or DHA; mean ± standard error of the mean * $P < 0.05$.

Discussion

Eicosapentaenoic acid and DHA are two common omega-3 fatty acids found in fish by-products which can be readily incorporated into glycerophospholipids and sphingolipids. Numerous studies have demonstrated that dietary supplementation with fish by-products increase the percentage of omega-3 fatty acids into the plasma membrane of cells (53, 69-73) and remodel lipid microdomains (74). However, recent data using artificial membranes have shown a tendency for glycerophospholipids containing DHA to be incorporated into lipid microdomains twice as efficiently as those having EPA (154), indicating that DHA may be preferentially incorporated into lipid microdomains. Yet, it is still unclear which individual omega-3 fatty acid plays a greater role in disrupting organization of microdomains. Most studies have reported that DHA has greater influence on lipid microdomain structure (54, 87, 96, 150, 151), despite the fact that DHA-containing glycerophospholipids are more ordered than those containing EPA (154, 155). A recent study reported that EPA and DHA equally disrupt lipid microdomains in immune cells (156). The present study investigated the effects of individual omega-3 fatty acids, EPA and DHA, on luteal cell fatty acid composition, spatial distribution of lipid microdomains, and lateral mobility of the FP receptor on the plasma membrane of cultured bovine luteal cells.

Results from experiment 1 show that luteal cells treated with fish oil had increased content of EPA and DHA as compared to cells cultured in control medium. The percentage of EPA and DHA in fish oil treated cells was comparable to the percentage of EPA and DHA in cells of corpora lutea collected from cows supplemented with fish meal (72, 153). Free fatty acids of EPA or DHA were both readily incorporated

into biological membranes of luteal cells as well, increasing in a dose-dependent manner. Neither of these fatty acids had a significant influence on abundance of other major long-chain fatty acids commonly found in biological membranes with the exception of arachidonic acid. While both EPA and DHA decreased the percentage of arachidonic acid at the highest dose examined (100 μM), only EPA was shown to be significant from control cells. Inclusion of fish meal in the diet of cows has been reported to decrease arachidonic acid in both endometrial and luteal tissue (69, 72). The decrease in arachidonic acid observed in whole animal studies was most likely due to the incorporation of EPA into reproductive tissues, which resulted in displacement of arachidonic acid.

Lipid microdomains consist of cholesterol that is tightly packed with sphingolipid, which does not integrate well into the disordered glycerophospholipid bilayer of the plasma membrane. This aggregation of cholesterol and sphingolipid causes the formation of distinct patches ranging in size from 10 to 200 nm (61). Clustering of lipid microdomains can be visualized through binding of fluorescently labeled cholera toxin B to monosialotetrahexosylganglioside and antibody cross-linking, resulting in distinct patches on the plasma membrane (157). It has been demonstrated that incorporation of fatty acids from fish by-product diminished microdomain clustering in bovine luteal cells *in vitro* (70) and *in vivo* (153). However, it was not clear from these previous studies which omega-3 fatty acid in fish by-products contributed to the disruption of lipid microdomain structure.

Experiment 2 investigated the influence of EPA and DHA on lipid microdomain structure. Data from experiment 2 show that cells treated with fish oil had decreased total

florescence intensity which is consistent with a previous study from this laboratory (70) When assessing an individual fatty acids, both EPA and DHA had a similar effect on microdomain structure in this cell type. Increasing the dose of both EPA and DHA from 1 to 100 μM resulted in a linear decline in total cell florescence intensity. These data are in agreement with several studies showing omega-3 fatty acids alter lipid microdomain structure (53, 154). The decrease in florescent intensity was most likely due to an increase in lipid microdomain size, leading to a decreased ability for cross-linking of cholera toxin B subunit. However, unlike the current study wherein culturing luteal cells in presences of individual omega-3 fatty acids or fish oil resulted in a decrease in total fluorescent intensity, other studies using immune cells treated with fish oil observed an increased intensity. Discrepancies between our data and previously reported studies regarding fluorescent intensity may be due to experimental methodology. In this study, live cells adhered to cover slips were used to determine spatial distribution of lipid microdomains, while other studies determined fluorescent intensity in fixed non-adherent cells. The adhesion of cells to cover slips may influence the cytoskeleton in EPA-, DHA-, and fish oil-treated cells, thereby affecting cross-linking of antibody to cholera toxin B, resulting in reduced fluorescent intensity. Furthermore, fixing of cells may influence the cytoskeleton of cells (158), leading to an apparent increase in fluorescent intensity in cells treated with fish oil.

Cholesterol plays a significant role in the formation and integrity of lipid microdomains. Early work using single particle tracking showed that these domains are approximately 200 nm in diameter and “trap” membrane proteins for several seconds. The removal of plasma membrane cholesterol disrupts these domains as indicated by

reduced number of domains and residence time of proteins within a domain (159). Mobility and residence time of FP receptors of bovine luteal cells are affected by fish oil treatment (70) and tissue obtained from fish meal supplemented animals (153). This suggests that in addition to cholesterol, omega-3 fatty acids may influence FP receptor membrane dynamics within the plasma membrane and microdomains of luteal cells.

Experiment 3 examined the influence of either EPA or DHA on lateral mobility of the FP receptor on the plasma membrane of luteal cells. Cells cultured in the presence of fish oil had increased micro- and macro-diffusion coefficients. In addition to increased diffusion rate of receptors, size of domains increased and residence time within domains decreased. These results on FP receptor dynamics for fish oil-treated cells are very similar to a previously reported study from this laboratory (70). Furthermore, both EPA and DHA had a significant influence on lateral mobility of FP receptor in a dose-dependent manner. Micro- and macro-diffusion coefficients were increased in cells cultured in 10 and 100 μM EPA and DHA compared to control cells, indicating both EPA and DHA have similar influences on FP receptor dynamics.

To our knowledge, this is the first study investigating the effects of individual omega-3 fatty acids, EPA and DHA, on bovine luteal cells. Plewes et al. (70) demonstrated fish oil increases both spatial distribution of lipid microdomains, as well as lateral mobility of the FP receptor of bovine luteal cells. Moreover, studies conducted *in vivo* demonstrated that dietary fish meal supplementation alters plasma fatty acid composition, luteal tissue fatty acid composition (72), and lipid microdomain integrity (153). Much work has been done to determine the beneficial effects of fish by-products on a number of cell types; however, the mechanistic role of omega-3 fatty acids is still

unknown. A number of studies have reported DHA has a greater impact on membrane dynamics. In bovine reproductive cells, both individual omega-3 fatty acids, EPA and DHA, have similar effects on disruption of lipid microdomains and lateral mobility of FP receptors. Furthermore, results from this study indicate this disruption of lipid microdomains and lateral mobility are correlated in a dose-dependent manner in these cells.

CHAPTER 5

INFLUENCE OF OMEGA-3 POLYUNSATURATED FATTY ACIDS FROM FISH OIL OR MEAL ON THE STRUCTURE OF LIPID MICRODOMAIN IN BOVINE LUTEAL CELLS

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Contribution of Authors and Co-Authors

Manuscript in Chapter V

Author: Michele R. Plewes

Contributions: Developed and implemented the study design. Generated and analyzed data. Wrote first draft of the manuscript.

Co-Author: Patrick. D. Burns

Contributions: Helped conceive the study topic. Provided guidance on study design. Provided feedback data interpretation and manuscript preparation.

Co-Author: Peter. E Graham

Contributions: Helped conceive the study topic. Provided guidance on study design.

Co-Author: Jason Bruemmer

Contributions: Helped conceive the study topic. Provided guidance on study design.

Co-Author: Terry Engle

Contributions: Helped conceive the study topic. Provided guidance on study design.

Abstract

Biological membranes are composed of a lipid bilayer and proteins that form lipid microdomains. This study examined the effects of fish byproducts on lipid-protein interactions within lipid microdomains of bovine luteal cells. In experiments 1 and 2, luteal cells were prepared from corpora lutea (CL; n=4 to 8) collected at an abattoir. Experiment 1 was conducted to optimize ultrasonication in a detergent-free protocol for isolation of lipid microdomains. A power setting of 10 to 20% was effective in isolating lipid microdomains from bulk lipid. In experiment 2, cells were cultured in control medium or fish oil to determine influence of fish oil on distribution of lipid microdomain markers and prostaglandin F2α FP receptors. Cells treated with fish oil had a smaller percentage of microdomain markers and FP receptor in microdomains ($P < 0.05$). In experiments 3 and 4, cells were prepared from mid-cycle CL obtained from cows supplemented with corn gluten meal (n=4) or fish meal (n=4). Experiment 3 examined effects of dietary supplementation on distribution of lipid microdomain markers and FP receptor and experiment 4 on fatty acid composition within lipid microdomains. A smaller percentage of lipid microdomain markers and FP receptor was detected in microdomains of cells collected from fish meal supplemented animals ($P < 0.05$). In experiment 4, a greater percentage of omega-3 polyunsaturated fatty acids was detected in bulk lipid from fish meal supplemented cows ($P < 0.05$). Results show that fish byproducts influence lipid-protein interactions in lipid microdomains in bovine luteal cells.

Introduction

Prostaglandins (PG) are endogenous lipid mediators involved in numerous female reproductive functions, such as ovulation and corpus luteum (CL) function (160-162). These mediators are derived from arachidonic acid which is a long-chain polyunsaturated fatty acid predominantly found associated with membrane glycerophospholipids (163). Prostaglandin $F_{2\alpha}$ is a specific prostanoid involved in the regulation of the CL. The bovine CL is a steroidogenic gland responsible for the synthesis and secretion of progesterone which is essential for the maintenance of pregnancy. In a non-pregnant cow, $PGF_{2\alpha}$ is secreted from the uterus late in the estrous cycle (136, 160) which binds to the $PGF_{2\alpha}$ (FP) receptor, a membrane-bound, G-protein-coupled receptor (23, 164). The binding of $PGF_{2\alpha}$ to its receptor initiates a complex signaling cascade leading to regression of the gland (165-167). Often, early embryonic mortality occurs when a slow developing conceptus is unable to regulate $PGF_{2\alpha}$ secretion from the uterus, leading to termination of pregnancy (2, 142). Disrupting the structural integrity of lipid microdomains has been reported to attenuate G-protein signaling in neurons (168). Therefore, altering lipid microdomain structure in bovine luteal tissue may lead to diminished $PGF_{2\alpha}$ signaling, thereby increasing pregnancy rate.

Cholesterol and sphingolipids coalesce into microscopic domains ranging in diameter from 10 to 200 nm, referred to as lipid microdomains (60, 61). There are two major types of lipid microdomains that have been described for biological membranes (62, 63). Linear domains, often referred to as membrane or lipid rafts, are regions that are associated with the outer leaflet of the membrane and enriched with cholesterol and sphingolipids. Curvilinear domains, also known as caveolae, appear to be associated with

both outer and inner membrane that are enriched with cholesterol and the protein caveolin. Both of these domains are involved in cellular trafficking, endocytosis/exocytosis, maintenance of cholesterol homeostasis, and serve as signaling platforms during signal transduction (64-66). Additionally, both linear and curvilinear microdomains play a pivotal role in regulating G-protein-coupled receptor signaling in mammalian cells (67).

Dietary long-chain fatty acids, specifically omega-3 polyunsaturated fatty acids found in fish byproducts, can have a major impact on organization and function of biological membranes. There are three major omega-3 polyunsaturated fatty acids found in biological membranes which include α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These fatty acids can become incorporated into glycerophospholipids and sphingolipids, disrupting lipid microdomain structure (70, 153). A growing body of evidence in the literature has shown that omega-3 polyunsaturated fatty acids alter lipid-lipid interactions and displace proteins from microdomains (53, 74, 96, 150, 169-172) into bulk lipid regions. Furthermore, recent data using single particle tracking from our laboratory have shown that fish oil and dietary supplementation of fish meal affects membrane mobility of the FP receptor (70, 153). However, the influence of fish oil or dietary supplementation of fish meal on protein distribution within lipid microdomains of bovine luteal cells is unknown. We hypothesize that incorporation of omega-3 polyunsaturated fatty acids from fish oil and meal disrupts lipid-protein interaction and displaces proteins associated with lipid microdomains into bulk lipid membrane fractions. The objectives of the current study were to 1) investigate the effects of fish oil on protein distribution within lipid

microdomain in cultured bovine luteal cells *in vitro* and 2) effects of dietary supplementation on distribution of proteins associated with lipid microdomain and relative fatty acid composition in lipid microdomain and bulk lipid fractions of bovine CL *in vivo*.

Methods

Experiments

Two *in vitro* experiments were conducted to 1) optimize a detergent-free protocol for isolation of bovine luteal cell lipid microdomain (Exp. 1) and 2) examine the effects of fish oil on distribution of known markers associated with lipid microdomains and FP receptors in this cell type (Exp. 2). Bovine ovaries containing a CL (n = 4 to 8) were collected at a local slaughterhouse and transported to the laboratory at the University of Northern Colorado in 1× sterile PBS. Age of CL was estimated as described by Miyamoto et al. (152) and only mature mid-cycle CLs were used for all experiments.

Two *in vivo* experiments were conducted to determine the influence of dietary supplementation of fish meal on 1) disruption of known markers associated with lipid microdomain and FP receptor (Exp. 3) and 2) fatty acid content within lipid microdomains (Exp. 4) of bovine luteal cells. All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (Approval #13-4440A). Details regarding animal husbandry, experimental diets, and omega-3 polyunsaturated fatty acids within tissues have been previously reported (153). In brief, non-lactating beef cows of mixed breed were randomly assigned to corn gluten meal (n = 4) or fish meal (n = 4; SeaLac, Omega Protein) supplementation. Diets were isocaloric and isonitrogenous and met or exceeded NRC recommendations for non-lactating beef

cows (173). Animals were individually fed a grass hay diet (95% dry matter intake) and supplemented with either 5% (dry matter intake) corn gluten meal or fish meal for approximately 60 d to incorporate omega-3 polyunsaturated fatty acids into blood and reproductive tissues. Estrous cycles of cows were synchronized using two intramuscular injections of 25 mg $PGF_{2\alpha}$ at 14-d intervals so that ovaries would contain a mid-luteal phase CL (d 10 to 12) on approximately d 60 of the supplemental period. Ovaries bearing the CL were surgically removed as previously described (153) and placed in 1× sterile PBS on ice for transport to the laboratory at the University of Northern Colorado.

Primary luteal cell isolation and culture. Primary luteal cell cultures were prepared using sterile techniques as previously described (70) for tissue obtained from a local slaughterhouse and dietary supplemented animals. In brief, the CL was excised from the ovary and cut into 500 µm slices using a Stadie-Riggs hand-held microtome. Tissue slices were placed into T-25 culture flasks containing 5 mL dissociation medium (HBSS supplemented with 2000 units type 1 collagenase per gram of tissue and 0.1 % BSA). Following enzymatic digestion, cellular suspensions were removed and transferred to a sterile 15-mL conical tube. Cells were washed 3 times in 5 mL of sterile 1× PBS. Cells were centrifuged at 500 × g for 5 min at 4 °C and cell pellets were re-suspended in 10 mL of Ham's F12 culture medium, supplemented with 5% fetal bovine serum, 1× insulin-transferrin-selenium-supplement (Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (pH = 7.34), and placed on ice. Cell viability was determined using trypan blue exclusion using a hemocytometer. All cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Experiment 1: Optimization of Detergent-Free Preparation of Lipid Microdomains in Bovine Luteal Cells

Mixed luteal cells from each CL (n = 4) were grown in T-75 culture flasks until 80% confluence. Cells were moved from flasks using 0.025% trypsin (wt/vol) and 0.01% EDTA (wt/vol). Cells were washed several times in PBS using centrifugation at 500 × *g* for 5 min at 4 °C. After final centrifugation, PBS was removed and cell pellets were stored at -80 °C until subjected to lipid microdomain isolation using 0, 10, 20 or 50% power setting during the ultrasonication step of the protocol.

Experiment 2: Effects of Fish Oil on Distribution of Lipid Microdomain Marker, and FP Receptor in Bovine Luteal Cells

Mixed luteal cells from each CL (n = 4 to 8) were added to three T-75 culture flasks and grown to approximately 80% confluency. At 80% confluency, culture medium was removed and flasks were randomly assigned to receive BSA control medium (n = 2 flasks/CL) or 0.03% (vol/vol) fish oil supplemented medium (n = 1 flask/CL). Cells were incubated in assigned culture medium for an additional 72 h to allow incorporation of long-chain fatty acids from fish oil into membranes. Lipids in a commercial fish oil (Pharmavite, Mission Hills, CA) were pre-bound to BSA prior to the addition to culture. In brief, fish oil was added to culture medium containing 33 mg/mL fatty acid free BSA as described by Mattos et al. (88) Control medium was prepared as stated above without the addition of fish oil. After 72 h of incubation, one flask treated with BSA control

medium was treated for an additional 1 h with 10 mM beta-methylcyclodextrin (β-MCD) to remove membrane cholesterol, serving as a positive control for lipid microdomain disruption. Cells were removed from flasks as described in Exp. 1 and cell pellets were stored at -80 °C until lipid microdomain isolation.

Experiment 3: Effects of Dietary Supplementation on Distribution of Lipid Microdomain Markers and FP Receptor in Bovine Luteal Cells

Immediately following cell dissociation, isolated mixed luteal cells (1×10^8 cells/ CL) from supplemented animals were incubated in 15 mL conical tubes for 1 h in serum free Ham's F12 culture medium or medium containing10 mM β-MCD, removing membrane cholesterol. Cells were harvested as above and stored at -80 °C until isolation of lipid microdomains.

Experiment 4: Influence of Dietary Fish Meal Supplementation on Lipid Microdomain Fatty Acid Composition

Immediately following cell dissociation, isolated mixed luteal cells (1×10^8 cells/ CL) from supplemented animals were incubated in 15 mL conical tubes for 1 h in serum free Ham's F12 culture medium or medium containing10 mM β-MCD to remove membrane cholesterol. Cells were prepared as above and stored at -80 °C until isolation of lipid microdomains and subsequent fatty acid analysis.

Isolation of lipid microdomains from bulk lipid. All procedures were carried out on ice unless otherwise indicated. Cell pellets were re-suspended in 0.66 mL of 500

mM Na_2CO_3 buffer (pH 11) supplemented with protease inhibitor, transferred to 11 × 60 mm culture tubes, and incubated for 30 min. Following incubation, cells were homogenized with 10 strokes in a 3-mL hand-held Dounce homogenizer and subsequently passed through a 27-gauge needle 30 times. Cell lysates were then sonicated (Branson Ultrasonics Danbury, CT USA) 3 times at 20 s intervals using 0, 10, 20, or 50% power setting (Exp.1), 10% for Exp. 2, and 20% for Exp. 3 and 4. Six hundred sixty-six microliters of 90% sucrose prepared in 2- (*N*-morpholino) ethanesulfonic acid (MES) buffer (20% glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM MES, pH 6.5) were added to homogenized samples resulting in a final concentration of 45% sucrose. A discontinuous sucrose gradient was prepared by carefully overlaying 1.32 mL of 35 and 5% sucrose in MES buffer containing 250 mM $NaCO_3$. Samples were placed in a pre-cooled SW60 Ti hanging bucket rotor (Beckman Coulter) and centrifuged at 250,000 × g for 24 h at 4 °C. Following centrifugation, ten 400 µL fractions were carefully remove from top to bottom of sample, placed in 1.7 mL microcentrifuge tubes, and stored at -20 °C until subjected to western blotting, dot-blot, or protein quantification.

Western blotting analysis. Proteins were precipitated from sucrose using trichloroacetic acid (TCA) as described (174) prior to western blotting. In brief, 200 µL of 0.5 M TCA were added to a 200 µL aliquot from each fraction and incubated at 4 °C for 24 h. Following incubation, samples were centrifuge at 20,000 × g for 10 min at 4 °C. Supernatant was removed and protein was washed 2 times with 200 µL ice cold acetone at 4 °C. Acetone was removed and protein samples were placed on a dry heat bath at 90

°C for 30 s to remove excess acetone. For each sample, 30 µL of 1 × Laemmli loading buffer was added and placed in dry heat bath for 6 min at 100 °C.

Proteins from individual fractions were used to determine the effects of fish byproducts on lipid microdomain markers. Following validation of microdomains, lipid microdomain fractions (1, 2, 3, and 4) and bulk lipid fractions (5, 6, 7, 8 ,9 and 10) were pooled to determine the effects of fish byproducts on FP receptors distribution. Proteins were resolved using 10% SDS-PAGE. An equal volume (15 µL) of sample was loaded into wells of the gel and a constant voltage of 140 V was applied for approximately 60 to 90 min to separate proteins. Proteins were then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes at 25 V for 90 min. Transfer efficiency of proteins to the membranes were evaluated by Ponceau S staining prior to blocking. Membranes were blocked with TBS-T (10 mM Tris-HCl pH 7.4, 140 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk solution at room temperature for 1 h. Membranes were incubated in primary antibody (1:500) for 24 h at 4 °C for detection of lipid microdomain markers (caveolin-1; Santa Cruz Biotechnology; SC-894 and flotillin-2; Santa Cruz Biotechnology; SC-25507), bulk lipid (calnexin; Santa Cruz Biotechnology; SC-11397) or FP receptor (Cayman Chemical, Ann Arbor, Michigan, USA). Membranes were rinsed 3 times with 1× TBS-T for 5 min. Membranes were then incubated with appropriate horse radish peroxidase-linked secondary antibody (1:3000; Santa Cruz) for 1 h at room temperature. Then blots were again rinsed 3 times with 1× TBS-T for 5 min each. Chemiluminescent substrate (SuperSignal; Thermo Fisher Scientific) was applied per manufacturer's instructions. Blots were visualized using VersaDoc imaging system (Bio-Rad, Hercules, CA, USA) and the percent abundance of

immunoreactive protein in each fraction was determined using densitometry analysis in ImageJ. The percentage abundance of protein in lipid microdomains was calculated by summing fractions 1, 2, 3, and 4, then dividing by total. The percentage abundance of protein in bulk lipid was calculated by summing fractions 5, 6, 7, 8, 9, and 10, then dividing by total.

Dot-blot analysis of GM_1. Ganglioside, monosialotetrahexosylganglioside, (GM_1) was used as an additional lipid microdomain marker which is predominately associated with linear domains. Dot-blot methodology was used to visualize distribution of GM_1 within individual gradient fractions as described by Chabraoui et al. (175) with modification. In brief, PVDF membranes were activated with 100% methanol for 3 min and rinsed with water for 5 min. Membranes were placed on blotting apparatus and samples were applied as described by the manufacturer's protocol (Bethesda Research Laboratories, Gaithersburg, MD, USA). Following loading of samples, membranes were removed and placed into blocking solution (TBS-T and 5% non-fat milk) for 1 h at room temperature. Following blocking in milk, cholera toxin subunit B conjugated to horse radish peroxidase (2 μg/mL; Life Technologies, Carlsbad, CA) was added to blocking solution for 1 h at room temperature. Membranes were rinsed 3 times in TBS-T at 5 min intervals. After final wash, membranes were visualized and analyzed as described for western blotting.

Quantitation of protein. Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA) was performed following protein precipitation to determine the effects on sonication on protein distribution within fractions. Protein from each fraction was

reconstituted in 200 µL of Millipore water and assay was performed per manufacturer's protocol using microtiter plates.

Fatty acid analysis. Lipid microdomain and bulk lipid fractions were pooled (200 µL) prior to lipid extraction. Lipids from pooled fractions were extracted using the Folch procedure (176). Extracted lipids were then methylated using a direct methylation protocol as previously described (72). Fatty acid composition of within each pooled sample was determined by GLC using an Agilent 7890A Series® gas chromatograph (Wilmington, DE) with a MS detector. The instrument was equipped with a 30-m × 0.20-mm (i.d.) fused silica capillary column (Supelcowax10; Supelco Inc., Bellefonte, PA, USA). Running conditions for GLC-MS were as previously described (153). Standard fatty acid methyl ester mixtures were used to calibrate the GLC system using reference standard GLC 68-D (Nu-Chek Prep, Inc. Waterville, MN, USA). Palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, α-linolenic acid, arachidonic acid, EPA, and DHA were identified by comparing the MS analysis and relative retention times of fatty acid methyl ester peaks of samples with those of standards. These peaks were then calculated as normalized area percentages of fatty acids.

Statistical Analysis

All data are reported as least square means ± standard error of the mean and unless otherwise stated, significance was declared at $P < 0.05$. For Exp. 1, effects of ultrasonication power setting on percent abundance of microdomain protein caveolin-1 and total protein were analyzed using 1-way analysis of variance. The model included CL, power setting (0, 10, 20 or 50%), fraction (microdomain vs bulk lipid), all possible interactions, and residual error as sources of variation. For Exp. 2, effects of fish oil on

percent abundance of microdomain markers (caveolin-1, flotillin-2, and GM_1), bulk lipid protein marker (calnexin), and FP receptor were analyzed using 1-way analysis of variance. The model included CL, treatment (control medium, 0.03% fish oil, or β-MCD), fraction (microdomain vs bulk lipid), all possible interactions, and residual error as sources of variation. Corpus luteum was considered a random variable in the model. Calculations were made using the mixed model procedure of SAS and means were compared by *t*-tests using the PDIFF option of SAS. For Exp. 3 and 4, effects of dietary supplementation on microdomain markers (caveolin-1, flotillin-2, and GM_1), bulk lipid protein marker (calnexin), FP receptor and fatty acid composition were analyzed using 1-way analysis of variance. The statistical model included dietary supplementation (corn gluten meal vs fish meal), cow, fraction (microdomain vs bulk lipid), all possible interactions, and residual error as sources of variation. Cow was considered a random variable in the statistical model. Calculations were made using the mixed model procedure of SAS and means were compared by *t*-tests using the PDIFF option of SAS.

Results

Experiment 1: Optimization of Microdomain Isolation in Bovine Luteal cells.

Lipid microdomains were isolated from bulk lipid using detergent-free isolation protocol to avoid potential artifacts often associated with using non-ionic detergents (177). This method uses sonication in a carbonate buffer to disrupt membrane lipids followed by separation of lipid microdomain from bulk lipid using a discontinuous sucrose gradient. Figure 32 shows the distribution of caveolin-1 and total protein following sonication amplitude power settings that ranged from 0 to 50%. Amplitude

power setting influenced distribution of caveolin-1 in bovine luteal cells. A power setting of 0% resulted in most of the caveolin-1 in fractions 2, 3 and 4 (Fig 32A and 32B). A power setting of 10 and 20% partitioned a greater percentage of caveolin-1 into fractions 1 and 2, thus indicating better disruption and separation of lipid microdomains from bulk lipid within membranes (Fig 32A and 32B). A setting of 50% amplitude completely disrupted membranes as indicated by equal distribution of caveolin-1 between lipid microdomain and in the bulk lipid (Fig 32A and 32B). However, distribution of total protein was not influenced by power setting (Fig 32C). Minimal protein was detected in lipid microdomains, while protein concentrations increased in bulk lipid.

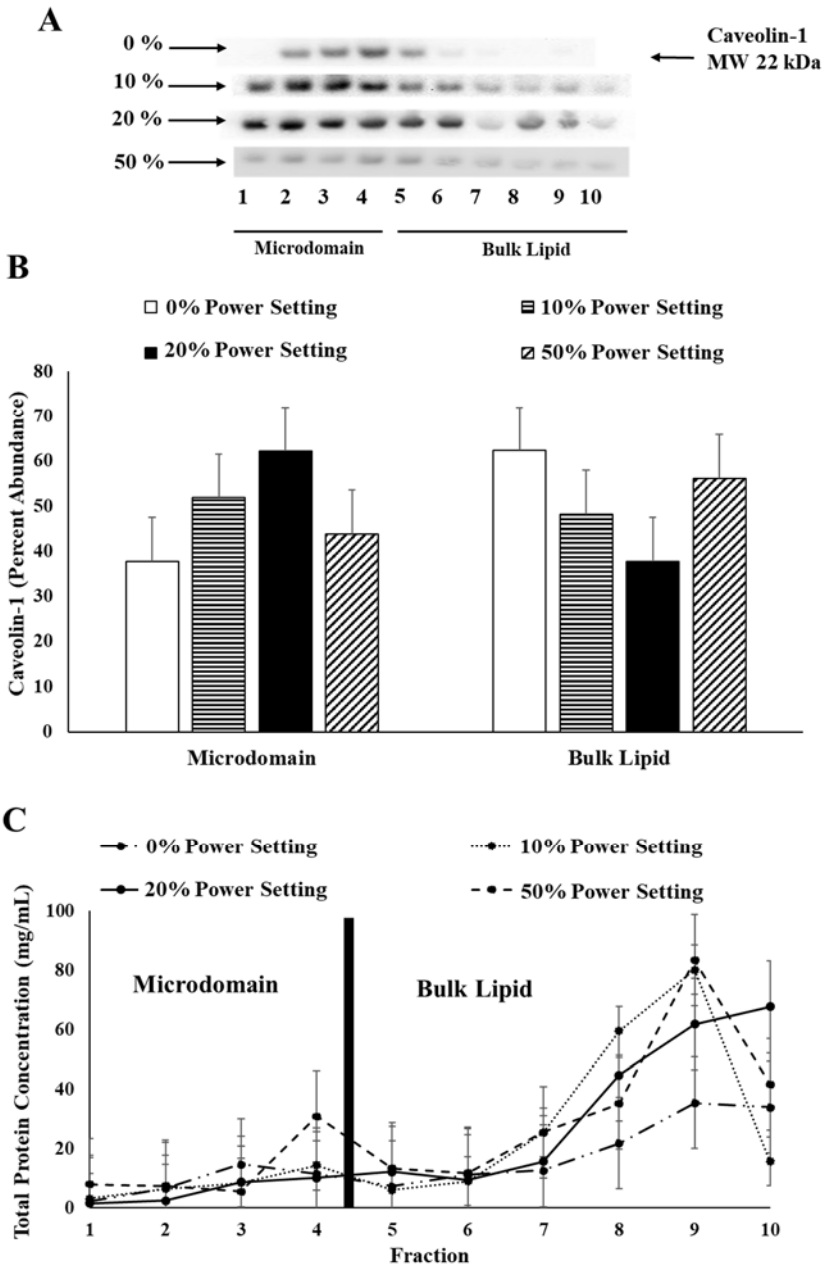

Figure 32. Optimization of detergent free preparation of lipid microdomains in bovine luteal cells. Panel A: Representative Western blot analysis showing effects of power setting on distribution of caveolin marker protein in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. 0% power setting (n = 4 open bar), 10% power setting (n = 4; horizontal bar), 20% power setting (n = 4; solid bar) or 10% power setting (n = 4; diagonal bar). Panel C: Quantitative analysis of total protein levels (mg/mL) within each fraction under the indicated conditions. 0 % power setting (n = 4; dash line with two dots), 10% power setting (n = 4; dotted line), 20% power setting (n = 4; solid line) or 50% power setting (n = 4; dashed line).

Experiment 2: Effects of Fish Oil on Distribution of Lipid Microdomain Markers and, FP Receptor in Bovine Luteal Cells In Vitro.

Caveolin-1 is a protein required for the formation of caveolae lipid microdomains within the plasma membrane found in most cell types (178). In BSA control cells, caveolin-1 abundance was greater within lipid microdomains than in bulk lipid ($P < 0.05$; Fig 33A and 33B). However, for cells treated with fish oil, distribution of caveolin-1 was greater in bulk lipid when compared to BSA control treated cells ($P < 0.05$). Similar to fish oil treatment, removal of membrane cholesterol with 10 mM β-MCD, shifted caveolin-1 abundance into the bulk lipid when compared to BSA control treated cell ($P < 0.05$; Fig 33A and 33B).

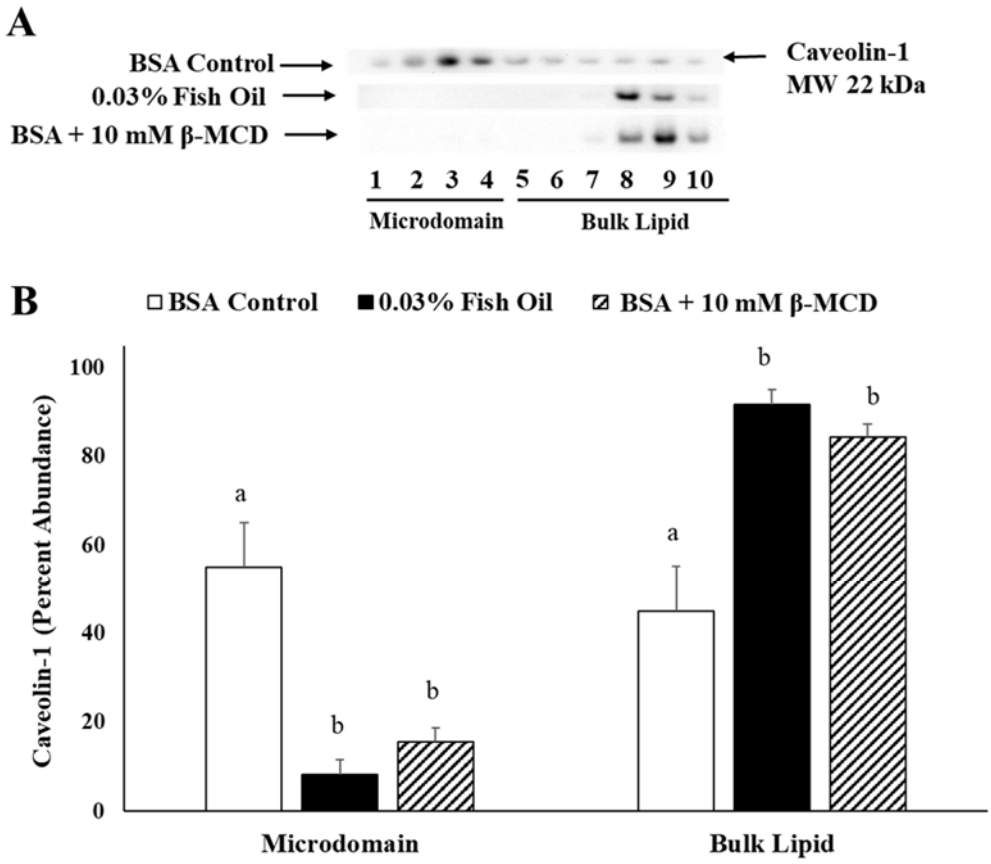

Figure 33. Effects of fish oil on distribution of caveolin, a protein associated with lipid microdomain in bovine luteal cells *in vitro*. Panel A: Representative Western blot analysis showing the effects of fish oil on caveolin distribution at 10% power setting in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. BSA control (n = 8; open bar), 0.03% fish oil (n = 6; solid bar) or BSA control subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; diagonal bar). Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$.

Flotillin-2 is a membrane protein that assists in the formation of linear lipid microdomains in the plasma membrane of all mammalian cell types (179). In control cells, flotillin-2 abundance was greater within lipid microdomain fractions than in bulk lipid (Fig 34A and 34B; $P < 0.05$). Similar to caveloin-1, distribution of flotillin-2 for cells treated with fish oil was greater in bulk lipid when compared to control treated cells

($P < 0.05$). Moreover, 10 mM β-MCD shifted flotillin-2 into the bulk lipid when compared to control cell (Fig 34A and 34B; $P < 0.05$).

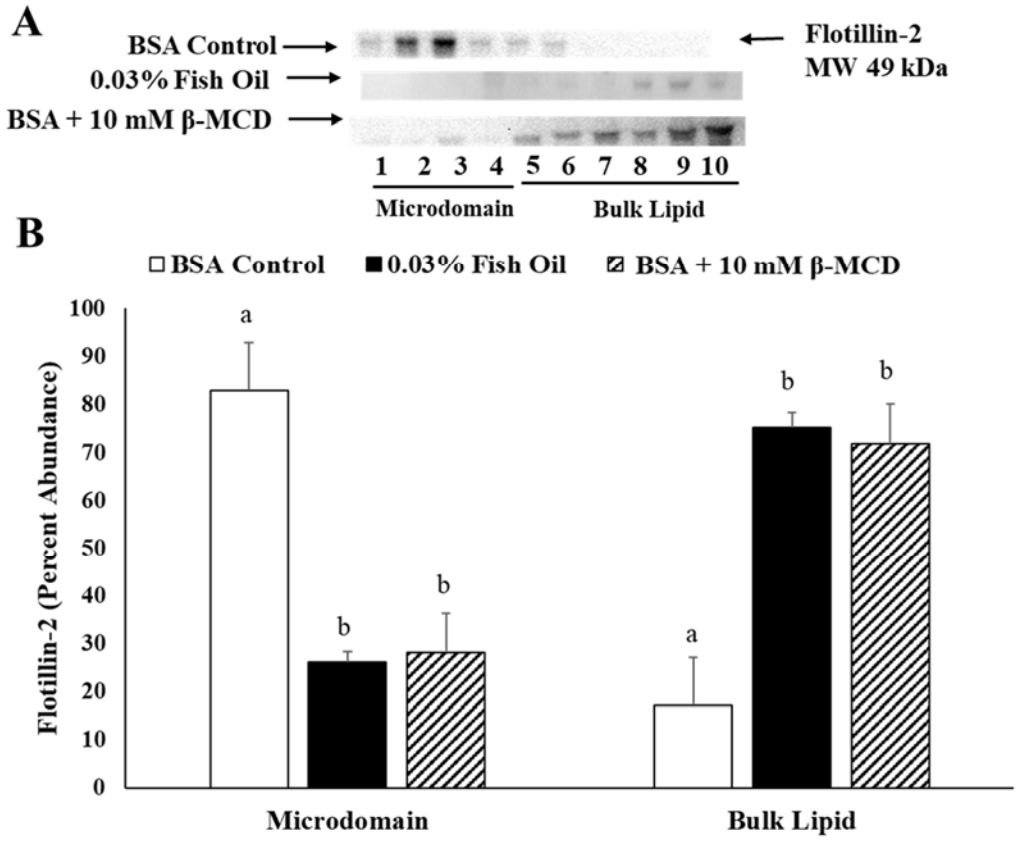

Figure 34. Effects of fish oil on distribution of flotillin, a protein associated with lipid microdomain in bovine luteal cells *in vitro*. Panel A: Representative Western blot analysis showing the effects of fish oil on caveolin distribution at 10% power setting in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. BSA control (n = 8; open bar), 0.03% fish oil (n = 6; solid bar) or BSA control subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; diagonal bar). Panel C: Representative Western blot analysis of the effects of fish oil on flotillin distribution at 10% power setting in bovine luteal cells. Panel D: Quantitative results from Western blot analysis. BSA control (n = 8; open bar), 0.03% fish oil (n = 6; solid bar) or BSA control subsequently treated with 10 mM β-MCD (n = 4; diagonal bar). Within pooled fraction (microdomain or bulk lipid), means with different letters^{ab} differ significantly; $P < 0.05$.

Linear lipid microdomains are composed of cholesterol and sphingolipids, such as ganglioside, GM_1 (180). In the control cells, GM_1 was greater within lipid microdomains

than in bulk lipid fractions (Fig 35; $P < 0.05$). However, treating cells with fish oil shifted GM_1 from lipid microdomain fractions into bulk lipid fractions when compared to control cells ($P < 0.05$). Additionally, removal of membrane cholesterol with β-MCD, shifted GM_1 into bulk lipid fractions in bovine luteal cells ($P < 0.05$).

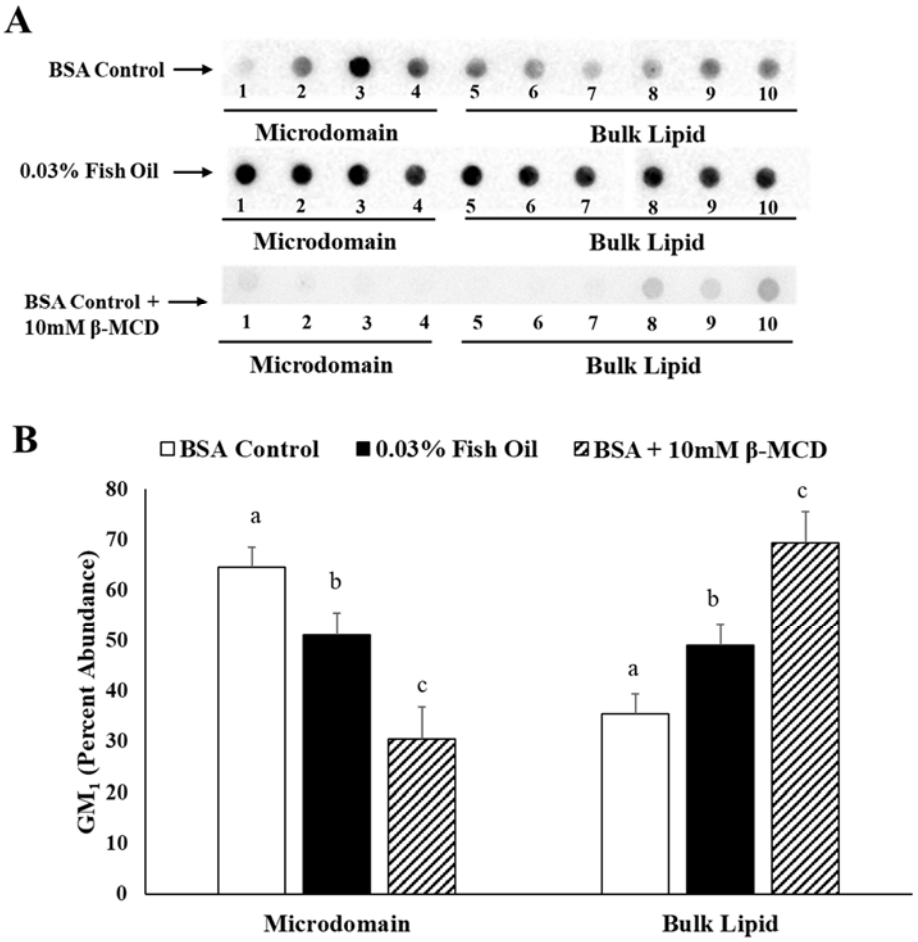

Figure 35. Effects of fish oil on distribution of lipid microdomain glycolipid in bovine luteal cells *in vitro*. Panel A: Representative Dot blot analysis showing the effects of fish oil on monosialotetrahexosylganglioside (GM_1) distribution at 10% power setting in bovine luteal cells. Panel B: Quantitative results from Dot blot analysis. BSA control (n = 4; open bar), 0.03% fish oil (n = 4; solid bar) or BSA control subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; diagonal bar. Within pooled fraction (microdomain or bulk lipid), means with different letters^{abc} differ significantly; $P < 0.05$.

Calnexin is a membrane-bound molecular chaperone that assists in the folding of Asn-linked glycoproteins that pass through the endoplasmic reticulum (181) and was used as a marker protein for bulk lipid. This protein was detected in the bulk lipid fractions for both control and fish oil treated cells and did not differ between the two treatments (Fig 36A and 36B; $P > 0.05$).

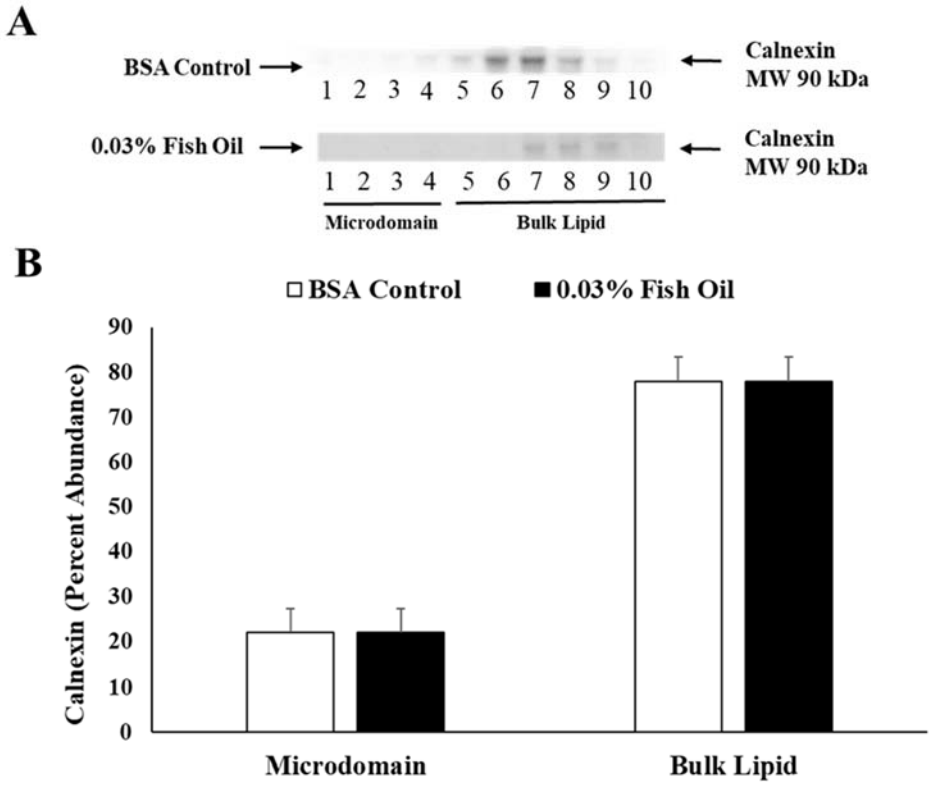

Figure 36. Effects of fish oil on distribution of Calnexin expression in bovine luteal cells *in vitro*. Panel A: Representative Western blot analysis showing the effects of fish oil on calnexin distribution at 10% power setting in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. BSA control (n = 4; open bar) or 0.03% fish oil (n = 4; solid bar). Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$.

Microdomains are known to play a critical role in the compartmentalization and modulation of G-protein signaling (68, 182-184). G-protein coupled FP receptor was

greater in lipid microdomain fractions as compared to bulk lipid fractions for control cells (Fig 35C and 35D; $P < 0.05$). Incubation of luteal cells in fish oil decreased the abundance of FP receptor in the microdomain fractions when compared to control cells (Fig 35C and 35D; $P < 0.05$). Removal of cholesterol did not influence distribution of FP receptors in lipid microdomain fractions when compared to control cells ($P > 0.05$).

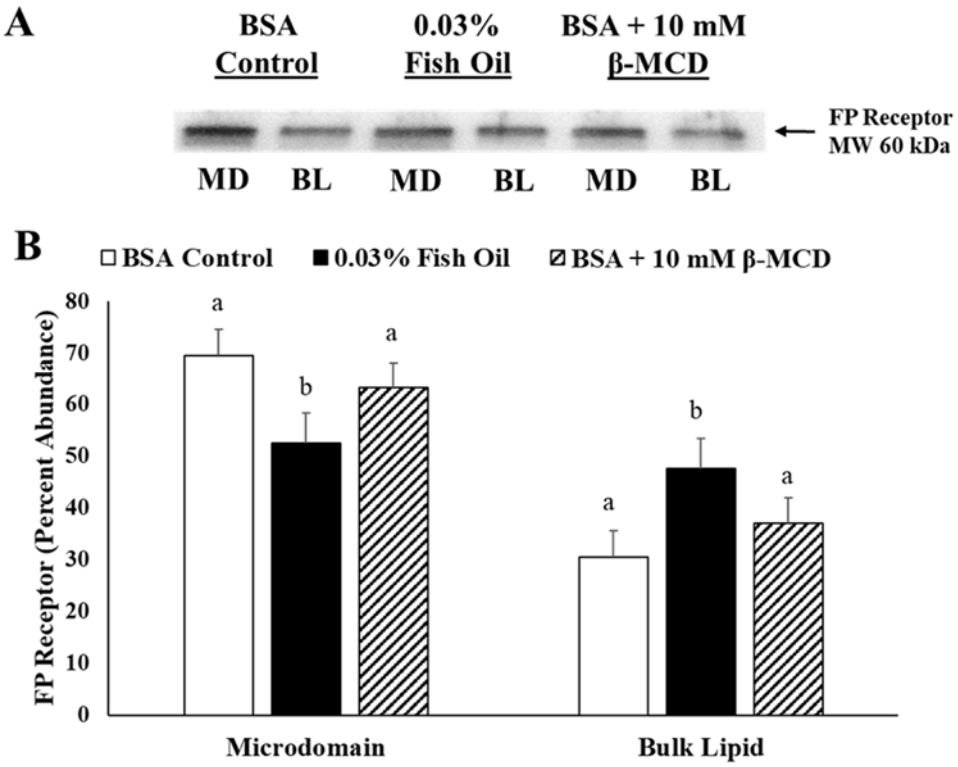

Figure 37. Effects of fish oil on distribution of FP receptor expression in bovine luteal cells *in vitro*. Panel A: Representative Western blot analysis showing the effects of fish oil on prostaglandin $F_{2\alpha}$ (FP) receptor distribution at 10% power setting in bovine luteal cells. Sucrose fractions 1, 2, 3, and 4 were pooled representing microdomain (MD) and sucrose fraction 5, 6, 7, 8, 9, and 10 were pooled representing bulk lipid (BL). Panel B: Quantitative results from Western blot analysis. BSA control (n = 4; open bar), 0.03% fish oil (n = 4; solid bar) or BSA control subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; diagonal bar). Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$.

Experiment 3: Effects of Dietary Supplementation of Fish Meal on Distribution of Lipid Microdomain Markers and FP Receptor within Lipid Microdomains of Bovine Luteal Cells In Vivo.

Caveolin-1 distribution in lipid microdomains and bulk lipid of mixed luteal cells obtained from corn gluten meal and fish meal supplemented cows is shown in Figure 38. Caveolin-1 had greater abundance within lipid microdomains when compared to bulk lipid from cells collected from corn gluten meal supplemented animals (Fig 38A and 38B; $P < 0.05$). Removal of cholesterol from tissue obtained from corn gluten meal supplemented animals shifted distribution of caveloin-1 into the bulk lipid fraction ($P < 0.05$). Caveolin-1 was greater in the bulk lipid fractions from luteal cells obtained from fish meal supplemented animals when compared to corn gluten meal treated animals ($P < 0.05$). Treating luteal cells of tissue obtained from fish meal supplemented cows with 10 mM β-MCD had no influence on caveolin-1 distribution untreated cells collected from fish meal supplemented animal (Fig 38A and 38B; $P > 0.05$).

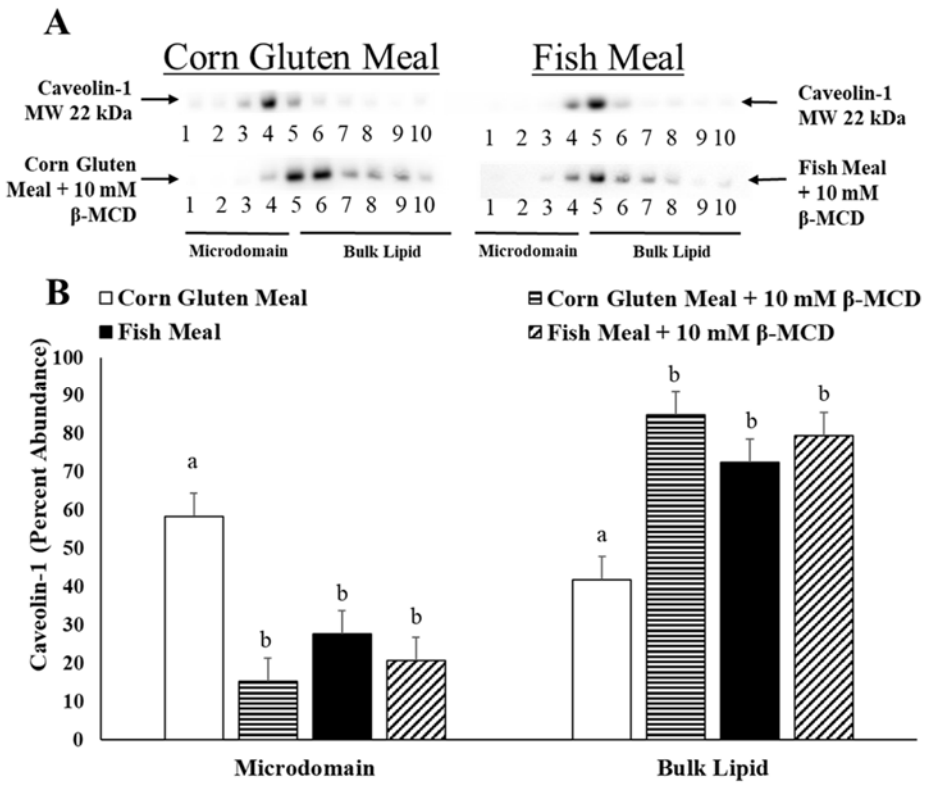

Figure 38. Effects of dietary supplementation on distribution of microdomain protein, caveolin, in bovine luteal cells *in vivo*. Panel A: Representative Western blot analysis showing the effects of dietary supplementation of fish meal on caveolin distribution at 20% power setting in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. Corn gluten meal (n = 4; open bar), cells obtain from same corn gluten meal supplemented corpus luteum subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; horizontal bar), fish meal (n = 4; solid bar), or cells obtain from same fish meal supplemented corpus luteum subsequently treated with 10 mM β-MCD (n = 4; diagonal bar). Within pooled fraction (microdomain or bulk lipid), means with different letters^{ab} differ significantly; $P < 0.05$.

Flotillin-2 distribution in lipid microdomains and bulk lipid of mixed luteal cells obtained from corn gluten meal and fish meal supplemented cows is shown in Figure 5. Similar to caveloin-1 observation, flotillin-2 was more abundant in luteal membrane bulk lipid fractions from animals supplemented with fish meal ($P < 0.05$; Fig 39A and 39B).

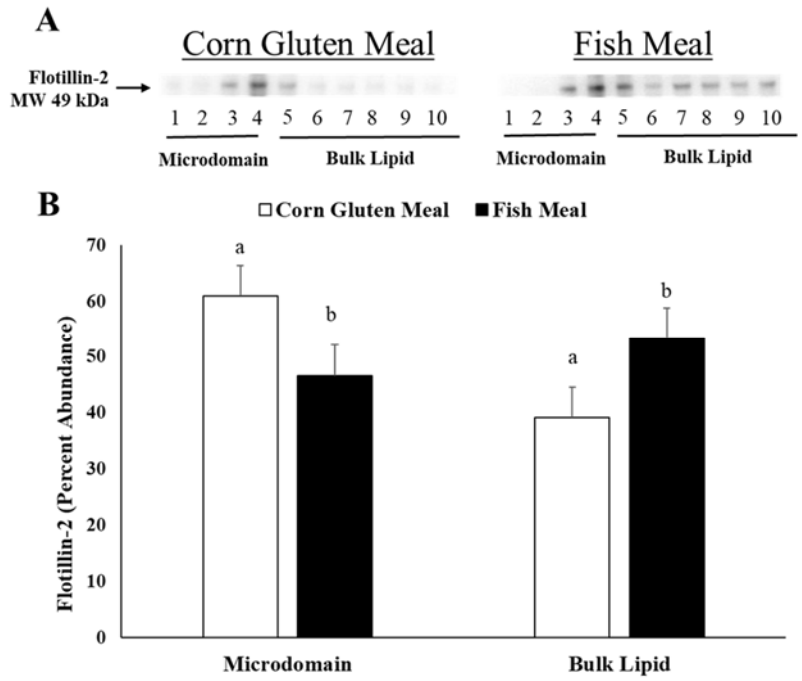

Figure 39. Effects of dietary supplementation on distribution of microdomain protein, flotillin, in bovine luteal cells *in vivo*. Panel A: Representative Western blot analysis showing the effects of dietary supplementation of fish meal on flotillin distribution at 20% power setting in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. Corn gluten meal (n = 4; open bar) or fish meal (n = 4; solid bar). Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$.

Distribution of GM_1 in lipid microdomains and bulk lipid of cells from luteal tissue obtained for corn gluten meal and fish meal supplemented animals is shown in Figure 40. GM_1 was greater within lipid microdomains fractions, than in bulk lipid for mixed luteal cells obtained from tissue collected from corn gluten meal supplemented animals ($P < 0.05$; Fig 40A and 40B). However, dietary supplementation of fish meal shifted this distribution of GM_1 from lipid microdomain fractions into bulk lipid fractions when compared to cells obtained from corn gluten meal supplemented animals ($P < 0.05$; Fig 40A and 40B). Treating luteal cells with 10 mM β-MCD had no influence on the

distribution of GM_1 for cells obtained from either corn gluten meal or fish meal supplemented animals ($P > 0.05$; Fig 40A and 40B).

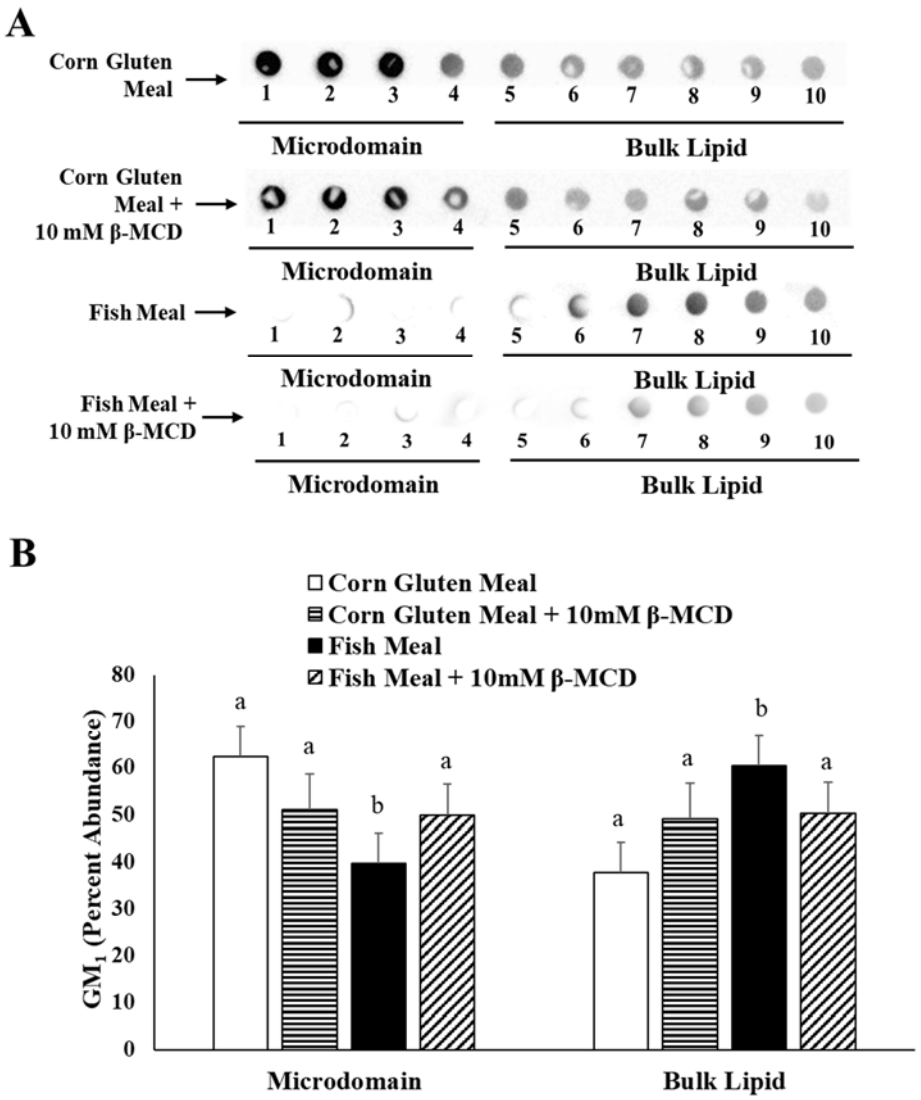

Figure 40. Effects of dietary supplementation on distribution of lipid microdomain glycolipid in bovine luteal cells *in vivo*. Panel A: Representative Dot-blot analysis showing the effects of dietary supplementation of fish meal on monosialotetrahexosylganglioside (GM_1) distribution at 20% power setting in bovine luteal cells. Panel B: Quantitative results from Dot blot analysis. Corn gluten meal (n = 4; open bar), cells obtain from same corn gluten meal supplemented corpus luteum subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; horizontal bar), fish meal (n = 4; solid bar), or cells obtain from same fish meal supplemented corpus luteum subsequently treated with 10 mM β-MCD (n = 4; diagonal bar). Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$.

Distribution of calnexin in lipid microdomains and bulk lipid from luteal cells obtained from corn gluten meal and fish meal supplemented cows is shown in Figure 41. Greater distribution of calnexin was observed in bulk lipid. Dietary supplementation had no influence on distribution of calnexin in either lipid microdomains or bulk lipid ($P > 0.05$; Fig 41B).

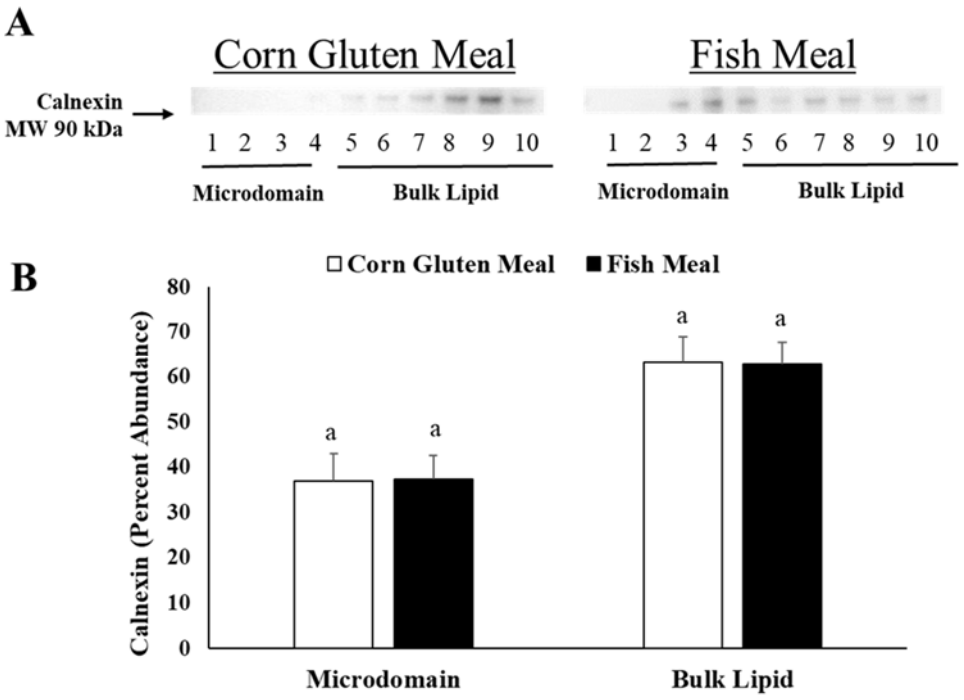

Figure 41. Effects of dietary supplementation on distribution of Calnexin and FP receptor in bovine luteal cells *in vivo*. Panel A: Representative Western blot analysis showing the effects of dietary supplementation of fish meal on calnexin distribution at 20% power setting in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. Corn gluten meal (n = 4; open bar) or fish meal (n = 4; solid bar). Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$.

Distribution of FP receptor in lipid microdomains and bulk lipid from luteal cells obtained from corn gluten meal and fish meal supplemented cows is shown in Figure 42. The distribution of FP receptor was greater within lipid microdomain fractions when

compared with bulk lipid fractions for luteal cells that were obtained from corn gluten meal supplemented animals ($P < 0.05$; Fig 42A and 42B). Dietary supplementation of fish meal shifted the distribution of the receptor from lipid microdomain fractions into bulk lipid fractions when compared to cells obtained from corn gluten meal supplemented animals ($P < 0.05$; Fig 42A and 42B). Treating luteal cells with 10 mM β-MCD had no influence in the distribution of FP receptor for tissue obtained corn gluten meal supplemented cows ($P > 0.05$; Fig 42A and 42B). However, β-MCD shifted receptor from bulk lipid into microdomain fractions for cells obtained from fish meal supplemented cows ($P < 0.05$; Fig 42A and 42B).

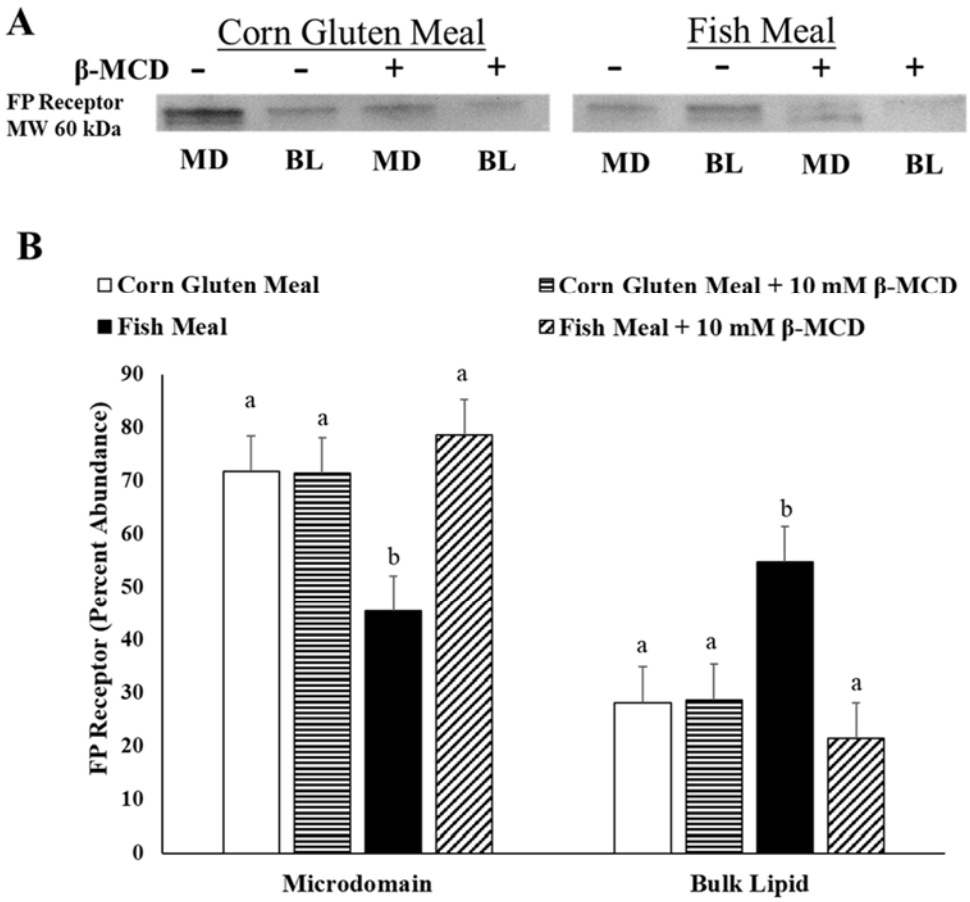

Figure 42. Effects of dietary supplementation on distribution of Calnexin and FP receptor in bovine luteal cells *in vivo*. Panel A: Representative Western blot analysis showing the effects of dietary supplementation of fish meal on prostaglandin $F_{2\alpha}$ (FP) receptor distribution at 20% power setting in bovine luteal cells. Microdomain was defined as fractions 1, 2, 3, and 4; bulk lipid was defined as fractions 5, 6, 7, 8, 9, and 10. Panel B: Quantitative results from Western blot analysis. Corn gluten meal (n = 4; open bar), cells obtain from same corn gluten meal supplemented corpus luteum subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; horizontal bar), fish meal (n = 4; solid bar), or cells obtain from same fish meal supplemented corpus luteum subsequently treated with 10 mM β-MCD (n = 4; diagonal bar). Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$.

Experiment 4: Effect of Dietary Supplementation of Fish Meal on Relative Composition of Long-Chain Fatty Acids in Microdomain and Bulk Lipid.

There was no effect of dietary supplementation, domain, or supplementation × domain interaction on fatty acid content of palmitic, palmitoleic, stearic, oleic, linoleic or arachidonic acids ($P > 0.05$). Furthermore, treating cells with β-MCD had no effect on distribution of these long-chain fatty acids in either microdomain or bulk lipid ($P > 0.05$). Figure 8 shows the effects of dietary supplementation and β-MCD treatment on relative composition of omega-3 polyunsaturated fatty acids in microdomain and bulk lipid. There was no effect of dietary supplementation on microdomain relative percent composition for α-linolenic acid ($P > 0.05$). However, cells obtained from fish meal supplemented animals had a lower α-linolenic acid in bulk lipid fraction when compared to corn gluten meal supplemented animals ($P < 0.05$). Additionally, luteal cells subsequently treated with 10 mM β-MCD for both supplements groups had a decreased α-linolenic acid in both lipid microdomain and bulk lipid fractions ($P < 0.05$; Fig 43A). Luteal cell content of EPA in the lipid microdomain fraction did not differ between cows supplemented with corn gluten meal and those supplemented with fish meal ($P > 0.05$; Fig 43B). However, EPA in cells obtained from fish meal supplemented animals was greater in the bulk lipid fraction when compared to corn gluten meal supplemented animals ($P < 0.05$; Fig 43B). There were no effects on lipid microdomain EPA content in corn gluten supplemented cells subsequently treated with 10 mM β-MCD ($P > 0.05$; Fig 43B). However, removal of cholesterol from luteal cells collected from fish meal supplemented animals resulted in increased EPA content in lipid microdomains ($P <$

0.05; Fig 43B). Similar to EPA, there was no effect of dietary supplementation on luteal cell content of DHA in microdomain fraction ($P > 0.05$; Fig 43C). However, luteal cells collected from fish meal supplemented animals had greater DHA in bulk lipid fraction when compared to cells collected from corn gluten meal supplemented animals ($P < 0.05$; Fig 43C). There were no effects on lipid microdomain DHA content in corn gluten supplemented cells subsequently treated with 10 mM β-MCD ($P > 0.05$; Fig 43C). However, as observed with EPA, removal of cholesterol from luteal cells collected from fish meal supplemented animals resulted in increased DHA content in lipid icrodomains ($P < 0.05$; Fig 43C).

Table 2: Effects of dietary supplementation on relative composition of microdomain and bulk lipid luteal long-chain fatty acids

	Corn Gluten Meal		**Corn Gluten Meal + 10 mM β-MCD**	
Fatty Acid	Microdomain	Bulk Lipid	Microdomain	Bulk Lipid
16:0	10.97 ± 3.35[a]	6.91 ± 3.35[a]	10.16 ± 3.35[a]	7.82 ± 3.35[a]
16:1	1.12 ± 0.40[a]	1.04 ± 0.40[a]	0.89 ± 0.40[a]	1.82 ± 0.40[a]
18:0	12.13 ± 2.71[a]	12.23 ± 2.71[a]	13.35 ± 2.71[a]	11.28 ± 2.71[a]
18:1	7.46 ± 1.87[a]	5.42 ± 2.71[a]	7.03 ± 2.71[a]	6.27 ± 2.71[a]
18:2	4.72 ± 1.83[a]	1.44 ± 1.83[a]	6.33 ± 1.83[a]	5.49 ± 1.83[b]
20:4	1.96 ± 0.74[a]	0.74 ± 0.74[a]	3.45 ± 0.74[a]	4.58 ± 0.74[b]
	Fish Meal		**Fish Meal + 10 mM β-MCD**	
Fatty Acid	Microdomain	Bulk Lipid	Microdomain	Bulk Lipid
16:0	13.25 ± 3.35[a]	10.98 ± 3.35[a]	16.07 ± 3.35[a]	12.43 ± 3.35[a]
16:1	1.63 ± 0.40[a]	2.42 ± 0.40[b]	0.11 ± 0.40[a]	0.38 ± 0.40[a]
18:0	15.23 ± 2.71[a]	12.27 ± 2.71[a]	19.95 ± 2.71[b]	18.60 ± 2.71[a]
18:1	8.69 ± 2.71[a]	5.41 ± 2.71[a]	9.19 ± 2.71[a]	4.52 ± 2.71[a]
18:2	6.03 ± 1.83[a]	2.043 ± 1.83[a]	6.06 ± 1.83[a]	6.36 ± 1.25[a]
20:4	2.13 ± 0.74[a]	0.98 ± 0.74[a]	2.98 ± 0.74[a]	1.10 ± 0.74[a]

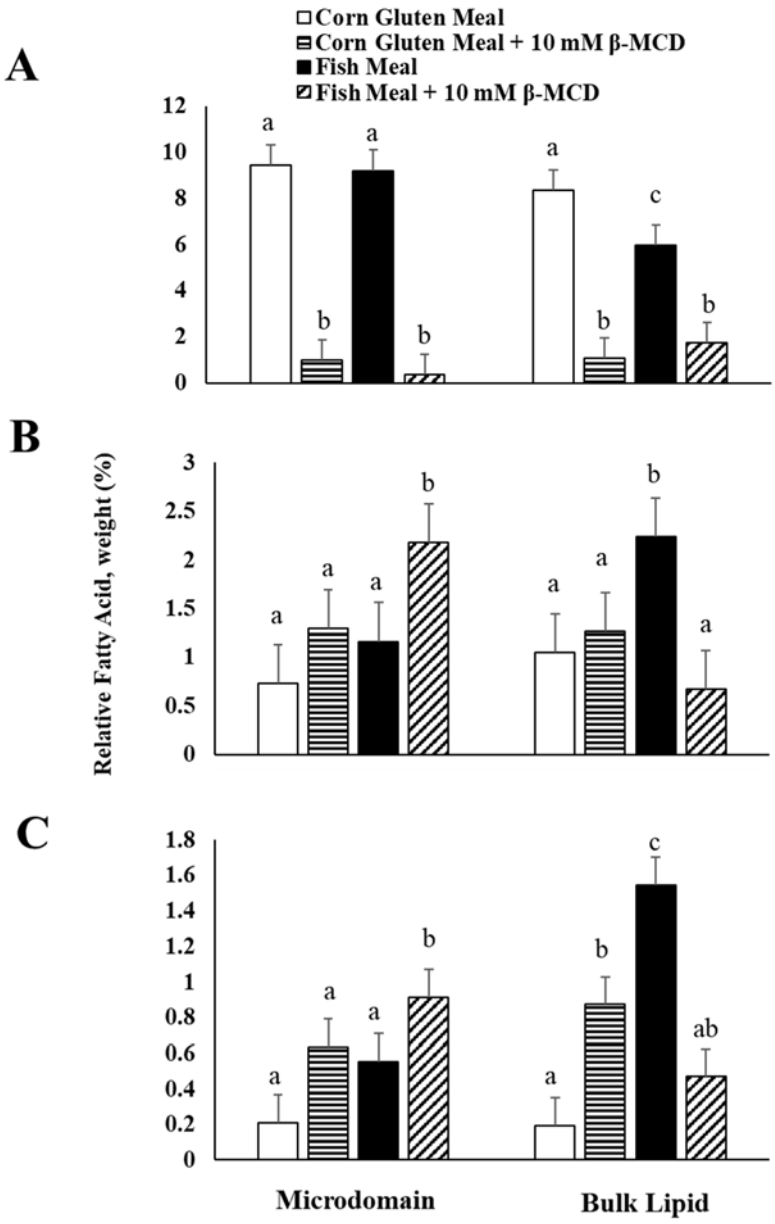

Figure 43. Effects of dietary supplementation on relative composition of microdomain and bulk lipid luteal long-chain fatty acids *in vivo*. Panel A: α-Linolenic acid; Within pooled fraction (microdomain or bulk lipid), means with different letters[abc] differ significantly; $P < 0.05$. Panel B: Eicosapentaenoic acid; Within pooled fraction (microdomain or bulk lipid), means with different letters[ab] differ significantly; $P < 0.05$. Panel C: Docosahexaenoic acid; Within pooled fraction (microdomain or bulk lipid), means with different letters[abc] differ significantly; $P < 0.05$. Corn gluten meal (n = 4; open bar), cells obtain from same corn gluten meal supplemented corpus luteum subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD; n = 4; horizontal bar), fish meal (n = 4; solid bar), or cells obtain from same fish meal supplemented corpus luteum subsequently treated with 10 mM β-MCD (n = 4; diagonal bar).

Discussion

We have conducted recent studies that show both fish oil and dietary supplementation of fish meal alters lipid microdomain structure and affects mobility of the FP receptor in bovine luteal cells (70, 153). Omega-3 polyunsaturated fatty acids that are found in fish byproducts have been shown to affect lipid microdomain structure (74, 95, 97). However, the influence of fish oil or dietary supplementation of fish meal on lipid-lipid and/or lipid-protein interactions within lipid microdomains is largely unknown within this cell type. Here, we report that fish byproducts (oil and meal) has a significant impact on distribution of known markers of lipid microdomains, FP receptor, and long chain-fatty acid composition within microdomains.

Lipid microdomains are well-ordered domains of cholesterol and sphingolipid giving rise to its unique biophysical property of insolubility in cold non-ionic detergents (185, 186). The insoluble microdomain fraction, being less dense due to greater lipid to protein, is separated using ultracentrifugation through sucrose density gradients from bulk lipid (177). However, the use of detergents can introduce artifacts (187). Detergent-free protocols that employ carbonate buffer and mechanical disruption through use of ultrasonication have been developed to alleviate this potential pitfall (177). Therefore, we opted to use detergent-free methodology for isolation of microdomains in the present study.

To our knowledge this is the first attempt to isolate lipid microdomains from bovine luteal cells. Markers of lipid microdomains should be present at the 5/35% sucrose interface which represents fractions 3 and 4 (128). Therefore, fractions 1, 2, 3, and 4 were defined as lipid microdomains and remaining fractions (5, 6, 7, 8, 9, and 10)

as bulk lipid. In this study using bovine luteal cells, a power setting of 10 and 20% during ultrasonication was effective in separating lipid microdomains from bulk lipid. The distribution of markers of lipid microdomains was similar to those reported in previous studies (128, 188, 189). Surprisingly, power setting did influence distribution of microdomain markers between our *in vitro* fish oil and *in vivo* dietary fish meal supplementation studies. A power setting of 10% was very effective in separating domains for *in vitro* experiments while 20% was required for *in vivo*. The differences between the two studies are difficult to explain, but may be due to membrane dynamics of cells cultured in flasks for several days compared to cells prepared from freshly digested tissue. It is also possible there are preferential differences in the incorporation of long-chain polyunsaturated omega-3 polyunsaturated fatty acids into various classes of lipid (e.g. glycerophospholipids or sphingolipids) during *in vitro* culture as compared to whole animal. Future studies are warranted for elucidating uptake and incorporation of long-chain polyunsaturated omega-3 fatty acids into biological membranes of bovine luteal cells.

Data from the current study support the working hypothesis that omega-3 polyunsaturated fatty acids in fish oil and fish meal affect lipid-protein interactions within lipid microdomains of bovine luteal cells. Inclusion of fish oil in luteal cell culture medium or cells obtained from cows supplemented with fish meal resulted in a greater percentage of microdomain structural markers (caveolin, flotillin, and GM_1) to be displaced into the bulk lipid. These data are in agreement with other studies wherein dietary supplementation or *in vitro* culture of omega-3 polyunsaturated fatty acids affect distribution of lipid microdomain markers (54, 74, 97, 169, 170, 190, 191). However,

neither fish oil nor dietary supplementation of fish meal affected distribution of bulk lipid marker (calnexin).

We also show that fish oil and dietary supplementation of fish meal not only affects lipid microdomain markers but also distribution of FP receptor. These results are in agreement with other studies showing omega-3 polyunsaturated fatty acids found in fish byproducts alter membrane receptor protein distribution (190-192). The FP receptor is a membrane-bound G-protein coupled-receptor present on the plasma membrane of both endothelial (25, 193) and steroidogenic luteal cells (24, 194) of the bovine CL. Single particle tracking studies of the FP receptor from our laboratory show that fish oil (70) and dietary supplementation of fish meal (153) increase lipid microdomain size and decrease residence time within microdomains. Decreased residence time of FP receptor within microdomains observed with supplementation of omega-3 polyunsaturated fatty acids may be a result of alterations between lipid-protein interactions as clearly shown in Exp. 2 and 3 of the current study. This alteration in membrane dynamics may influence luteal sensitivity to $PGF_{2\alpha}$.

The mechanism by which fish oil or meal alters lipid microdomain structure is largely unknown. It is possible alteration of fatty acid composition within biological membranes may influence lipid-protein interactions within microdomains as observed in the present study. The omega-3 polyunsaturated fatty acids are the most likely candidates that alter these interactions, as dietary supplementation did not influence long-chain saturated or monounsaturated fatty acids. Indeed, studies have shown that incorporation of EPA- and DHA-containing glycerophospholipids into biological membranes displace cholesterol from bulk lipid regions into lipid microdomains (195, 196). The poor affinity

for these long-chain polyunsaturated fatty acids with cholesterol creates EPA- and DHA-glycerophospholipid domains, or patches, which may be responsible for disruption of lipid microdomains. In the current study, supplementation of fish meal resulted in a greater relative percent of both EPA- and DHA-containing lipids in bulk fraction, as observed in previous studies (195, 196). Moreover, removal of membrane cholesterol using β-MCD resulted in a significant shift of lipids esterified with EPA and DHA into the lipid microdomain fractions, further supporting this hypothesis. Future work needs to be conducted to determine the specific roles cholesterol and omega-3 polyunsaturated fatty acids play on the integrity of lipid microdomain structure and function.

Early embryonic mortality continues to be a significant problem in animal agriculture (132, 197). One potential cause of early embryonic mortality is due to slow developing embryos failing to trigger maternal recognition of pregnancy (10). Fats have been added to the diet of beef and dairy cows as a means to improve reproductive performance. Inclusion of fish meal into the diet has been shown to increase pregnancy rates in beef (198) and lactating dairy cows (134, 135, 199, 200). The omega-3 polyunsaturated fatty acids in fish meal have been reported to influence uterine prostaglandin synthesis and secretion (40, 43), which is a possible mechanism for improved fertility. Another possible mechanism is that fish byproducts affect luteal membrane dynamics, specifically the lateral mobility of the bovine FP receptor (70, 153). Here we show that fatty acids in fish byproducts incorporate into the membranes disrupting the lipid-protein interactions within lipid microdomains; possibly explaining increased domain sizes and lateral mobility of the FP receptor as previously reported from our laboratory. Furthermore, we have recently shown that fish meal

supplementation reduces luteal sensitivity to intrauterine infusions of $PGF_{2\alpha}$ (114). Taken together, these results demonstrate that fish meal supplementation may be a novel approach for improving reproductive success. In summary, results from the present study demonstrate fish oil and dietary supplementation of fish meal affect lipid-protein interactions within lipid microdomains of bovine luteal cells. Additional experimentation is necessary to determine specific interactions with cholesterol and omega-3 polyunsaturated fatty acid containing lipids within these domains.

CHAPTER 6

EFFECT OF FISH MEAL SUPPLEMENTATION ON SPATIAL DISTRIBUTION OF LIPID MICRODOMAINS AND LATERAL MOBILITY OF PROSTAGLANDIN F2ALPH RECEPTORS ON BOVINE CORPUS LUTEUM PLASMA MEMBRANE

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Contribution of Authors and Co-Authors

Manuscript in Chapter VI

Author: Michele R. Plewes

Contributions: Developed and implemented the study design. Generated and analyzed data. Wrote first draft of the manuscript.

Co-Author: Patrick. D. Burns

Contributions: Helped conceive the study topic. Provided guidance on study design. Provided feedback data interpretation and manuscript preparation.

Co-Author: Peter. E Graham

Contributions: Helped conceive the study topic. Provided guidance on study design.

Co-Author: Jason Bruemmer

Contributions: Helped conceive the study topic. Provided guidance on study design.

Co-Author: Terry Engle

Contributions: Helped conceive the study topic. Provided guidance on study design.

Abstract

This study examined the effects of fish meal supplementation on spatial distribution of lipid microdomains and lateral mobility of prostaglandin $F_{2\alpha}$ (FP) receptors on cell plasma membranes of the bovine corpus luteum (CL). Beef cows were stratified by body weight and randomly assigned to receive a corn gluten meal supplement (n = 4) or fish meal supplement (n = 4) for 60 d to allow incorporation of fish meal derived omega-3 fatty acids into luteal tissue. Ovaries bearing the CL were surgically removed on d 10 to 12 post-estrus corresponding to approximately d 60 of supplementation. A 200 mg sample of luteal tissue was analyzed for fatty acid content using GLC. The remaining tissue was enzymatically digested with collagenase to dissociate individual cells from the tissue. Cells were cultured to determine effects of dietary supplementation on lipid microdomains and lateral mobility of FP receptors. Luteal tissue collected from fish meal supplemented cows had increased omega-3 fatty acids content ($P < 0.05$). Lipid microdomain total fluorescent intensity was decreased in dissociated luteal cells from fish meal supplemented cows ($P < 0.05$). Micro and macro diffusion coefficients of FP receptors were greater for cells obtained from fish meal supplemented cows ($P < 0.05$). In addition, compartment diameter of domains was larger while resident time was shorter for receptors from cells obtained from fish meal supplemented cows ($P < 0.05$). Data indicate that dietary supplementation with fish meal increases omega-3 fatty acid content in luteal tissue causing disruption of lipid microdomains. This disruption leads to increased lateral mobility of the FP receptor, increased compartment sizes, and decreased resident time which may influence prostaglandin signaling in the bovine CL.

Introduction

Luteinizing hormone from the anterior pituitary gland causes the release of the ovum from the follicle and differentiation of the theca and granulosa cells into small and large luteal steroidogenic cells, respectively (201-203). Luteal steroidogenic cells secrete progesterone which is essential for early pregnancy in mammals. In the non-pregnant cow, uterine prostaglandin (PG) $F_{2\alpha}$ is released late in the estrous cycle in a series of 5 to 8 pulses, causing regression of the corpus luteum (CL) allowing for return to estrus (136, 204, 205). Prostaglandin $F_{2\alpha}$ (FP) receptors are heterotrimeric G-protein coupled receptors located on steroidogenic luteal cells of the CL (206). Binding of $PGF_{2\alpha}$ to its receptor triggers a complex intracellular signaling cascade to initiate regression of the CL. In the pregnant female, the conceptus inhibits uterine synthesis and release of $PGF_{2\alpha,}$ preventing regression of the CL, allowing for continued secretion of progesterone and establishment of pregnancy (207). Loss of pregnancy can occur when a viable conceptus fails to adequately regulate $PGF_{2\alpha}$ secretion, leading to regression of the CL (208, 209). Therefore, diminishing or altering sensitivity of luteal cells to $PGF_{2\alpha}$ may prevent regression of the CL during early pregnancy.

The plasma membrane of mammalian cells is composed of a lipid bilayer which is highly dynamic. It also contains unique regions called lipid microdomains, ranging in size from 10 to 200 nm in diameter that are high in cholesterol and sphingolipids (210, 211). There are two distinct lipid microdomain structures associated with plasma membranes – linear domains referred to as lipid rafts and invaginated domains referred to as caveolae. These microdomains have been postulated to regulate vesicle trafficking/sorting, endocytosis of membrane-bound proteins, cholesterol homeostasis,

and serve as platforms to facilitate co-localization of intracellular signaling proteins during agonist-induced signal transduction (210). Numerous studies have shown that ligand-bound membrane receptors often coalesce into lipid microdomains following addition of an agonist resulting in the activation of intracellular signaling pathways (212-214). Therefore, altering lipid microdomain structure on the plasma membrane of luteal cells may reduce $PGF_{2\alpha}$ signaling in bovine CL.

Long-chain polyunsaturated fatty acids such as omega-3 fatty acids can be incorporated into glycerophospholipids and increase membrane fluidity (48-50). Changes in membrane order or fluidity has been reported to affect ligand affinity and subsequent ion flux for the acetylcholine receptor (51). In addition to altering membrane fluidity, these fatty acids have been reported to disrupt lipid microdomain composition, affect mobility of membrane-bound receptors, and decrease cell signaling (52-54). We recently reported that inclusion of fish meal in the diet of non-lactating beef cows increased blood plasma (43, 69, 72) and luteal (72) content of omega-3 fatty acids. Therefore, it is hypothesized that omega-3 fatty acids in fish meal will incorporate into the plasma membrane of luteal cells altering lipid microdomains and lateral mobility of FP receptors in cells of the bovine CL. The objectives of the current study were to examine the effects of fish meal supplementation on 1) plasma and luteal omega-3 fatty acid composition, 2) organization and spatial distribution of lipid microdomains on cells of the bovine CL and 3) lateral mobility of the membrane-bound FP receptors.

Methods

Animals and Tissue Collection

All animal procedures described herein were approved by the Colorado State University Institutional Animal Care and Use Committee (Approval # 13 - 4440A). Beef cows of mixed breeds were purchased at a local sale barn in Fort Collins, Colorado and housed at the Colorado State University Animal Reproduction Biotechnology Laboratory Foothills campus. Reproductive organs were palpated per rectum for presence of gross anatomical abnormalities (cystic follicles) and adhesions. Transrectal ultrasonography was performed on ovaries for presence of CL and uteri for absence of a fetus. Cows with adhesions, cystic follicles, absence of a CL, or pregnant were removed from the study.

Cows were stratified by BW and randomly assigned to receive corn gluten meal (n = 4; controls) or fish meal (n = 4; SeaLac, Omega Protein). Diets were delivered daily to cows at 2% BW on a dry matter intake basis that met or exceeded NRC requirements (215). The ration consisted of 95% mixed hay and 5% pelleted supplement of either fish meal or corn gluten meal. Diets were formulated to be isocaloric and isonitrogenous (Tables 3 and 4) and cows were fed for approximately 60 d to allow for adequate time to incorporate omega-3 fatty acids into blood and reproductive tissues in the fish meal supplemented animals. Cows were housed in a dry lot and were individually penned (3.7 × 3.0 m) between 0600 and 1000 each day to receive supplements and hay. After consumption of rations, cows were then turned out to have *ad libitum* access to water. Body weights were collected weekly to monitor changes in weight and diets were adjusted as needed.

Table 3. Chemical composition of mixed grass hay

Dry matter intake	%
Chemical Analysis	
Water Soluble Carbohydrates	8.7
Neutral Detergent Fiber	56.7
Acid Detergent Fiber	37.0
Simple Sugars	5.3
Starch	1.0
Non Fiber Carbohydrates	1.0
Crude Protein	15.2
Crude Fat	3.5

Table 4. Ingredient, chemical composition, and long-chain fatty acid profile of dietary supplementation

	Experimental Diet	
Item	Corn Gluten Meal	Fish Meal
Dry Matter Intake, %	5	5
Ingredient of Pelleted Supplement, %		
Fish meal	0	60.0
Corn Gluten meal	59.3	0
Wheat midds	19.2	17.4
Wheat – ground	7.3	7.8
Limestone	7.4	7.4
Molasses cane	4.0	4.0
Salt	1.3	1.3
Soybean oil-mixer	0.25	0.8
Magox	0.3	0.3
Monocal 16 21	0.25	0.25
Z_nSO_4	0.2	0.2
Se	0.1	0.1
M_nSO_4	0.1	0.1
$CuSO_4$	0.1	0.1
Vitamin A 30/3	0.1	0.1
Vitamin E 125	0.02	0.02
Vitamin D 0/30	0.02	0.01
Ranch-o-dine	0.02	0.02
Chemical Analysis		
Crude Protein, %	39.8	40.1
Degradable Intake Protein, %	29.5	29.9
Undegradable Intake Protein, %	33.7	34.2
Total Digestible Nutrients, %	68.8	69.6
Crude Fat, %	3.0	3.5
Fatty Acid Composition of Supplement, wt %		
Palmitic Acid (16:0)	14.3	26.4
Palmitoleic Acid (16:1)	1.5	8.7
Stearic Acid (18:0)	17.8	4.6
Oleic Acid (18:1)	38.8	12.5
Linoleic Acid (18:2)	2.2	11.6
Alpha-Linolenic Acid (18:3)	2.2	2.5
Arachidonic Acid (20:4)	<0.5	1.1
Eicosapentaenoic Acid (20:5)	<0.5	9.0
Docosahexaenoic Acid (22:6)	<0.5	8.9

Jugular blood samples were collected immediately before supplementation commenced and weekly thereafter to measure changes in plasma fatty acid composition. Samples were collected in 3-mL blood tubes containing 5.4 mg EDTA (BD Vacutainer, Becton and Dickson Co, Franklin Lakes, New Jersey, USA) and immediately placed on ice. Samples were centrifuged at 1500 × g for 15 min, after which plasma was then collected and stored at -80°C until GLC analysis.

Cows were administered 25 mg injections of $PGF_{2\alpha}$ (Lutalyse, Pharmacia & Upjohn Co, MI, USA) on d 36 and d 50 of the supplementation period to synchronize estrous cycles. Ovaries bearing the CL were surgically removed by standing flank procedure, as previously described (216) on d 10 to 12 post-estrus following the second $PGF_{2\alpha}$ injection (approximately d 60 of the supplementation period). After collection, the ovary was placed in 1× sterile PBS and transported on ice to the laboratory at the University of Northern Colorado. Superficial sterilization of the ovary was performed by immersing into a 70% ethanol solution.

Cell Preparation

Using sterile techniques under a laminar flow hood, the CL was removed from the ovary and a 200 mg sample of tissue was placed in a 1.7-mL micro centrifuge collection tube and stored at -80°C until GLC analysis. The remaining tissue was cut into 500 µm slices using a Stadie-Riggs microtome. Slices of tissue were placed into a 60-mm² Petri dish containing Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS) that was supplemented with 20 mM HEPES, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (pH = 7.34) until enzymatic digestion. Tissue was then placed in T-25 culture flasks containing 5 mL dissociation medium (HBSS supplemented

with 2000 units type 1 collagenase per g of tissue and 0.1 % BSA). Flasks were incubated in a water bath at 37°C with agitation for 45 min. Following incubation, the suspension was removed and transferred to a sterile 15-mL conical tube. Cells were washed 3 × in 5 mL of sterile 1× PBS. In brief, cells were centrifuged at 500 × *g* for 5 min at 4°C, the supernatant was removed, and cell pellets were re-suspended in fresh 5 mL 1× PBS. Following the final wash, the cell pellet was re-suspended in 10 mL of Ham's F12 culture medium, supplemented with 5% fetal bovine serum, insulin-transferrin-selenium-supplement (51300044; Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (pH = 7.34), and placed on ice. Fresh dissociation medium was added to the remaining undigested tissue and incubated with agitation for additional 45 min. Cells were collected, washed 3× with PBS as above, and then combined with the previous sample. Viability of the cells was determined using trypan blue exclusion, and cell number was estimated using a hemocytometer. Viability of the cell populations was 96 ± 1.8%.

Cell Culture

Cells were cultured in 35-mm round-bottom culture dishes with poly-D-lysine coated coverslips (MatTex Corporation, Calhoun GA, USA). To facilitate cellular adhesion to coverslips, biomatrix (BD matrigel matrix basement membrane, BD Biosciences MI, USA) was added to dishes immediately prior to addition of 5×10^4 viable cells/mL per manufacturer's protocol. Cells were maintained under an atmosphere of humidified air and 5% CO_2 at 37°C for approximately 12 h prior to lipid microdomain staining or FP receptor labeling.

Lipid Microdomain Staining and Analysis

Five dishes were prepared from each CL for lipid microdomain analysis. Two dishes were chosen at random and treated for 1 h with 10 mM beta-methyl cyclodextrin (β-MCD) to remove membrane cholesterol which leads to disruption of microdomains, thus serving as a positive control. Lipid microdomains were labeled as previously described (50).

Cells were viewed using a Zeiss confocal microscope equipped with a 40× water immersion objective (1.2 N.A). A 555 nm laser was used to excite Alexa-555 fluorophore, and emission of light was collected between 560 to 1000 nm. Approximately 30 cells were randomly selected from each dish, and 1 μm slice images were generated from bottom to top of each cell. Three-dimensional images were generated, and whole cell fluorescent intensity was determined using ImageJ software. A total of 366, 80, 578 and 117 dissociated cells were analyzed from tissue collected from controls, controls treated with β-MCD, fish meal supplemented cows, and fish meal supplemented cows treated with β-MCD, receptively.

Prostaglandin FP Receptor Labeling, Single Particle Tracking, and Analysis

Receptors were prepared for single particle tracking experiments using biotinylated antibody as previously described (50). Three to five dishes were prepared from each CL for single particle tracking. Biotin was conjugated to FP receptor rabbit polyclonal antibody (product no. 101802; Cayman Chemical, Ann Arbor, MI, USA) using a DSB-X biotin protein labeling kit (Life Technologies, Carlsbad, CA, USA) per manufacturer's instructions. Experiments were conducted using a Zeiss confocal

microscope equipped with a high speed camera (Hamamatsu Photonics, Japan). Ultraviolet light was used to excite quantum dots and emission of light was collected at 605 nm. Using 100× oil objective (N.A. = 1.4) and acquisition image size of 512 × 512 pixel (33.3 μm × 33.3 μm). Individual receptors were recorded for 29 s (30 fps) for a total of 870 frames per video. Receptors containing a minimum of 10 s of recordings were used for the final analysis.

Video Spot Tracker v08.01 software (Computer Integrated Systems for Microcopy and Manipulation, University of North Carolina, Chapel Hill, NC, USA) was used to generate individual receptor trajectories. The resolution was 0.07 μm/pixel, and the XY coordinates obtained in pixels from Spot Tracker were converted to μm^2. Receptor trajectories were visualized by plotting XY coordinates using Excel software and a mean square displacement (MSD) for each trajectory was calculated using the equations reported by Daumas (89). The MSD was then plotted against time. Micro diffusion coefficient was determined from the slope of the fitted line equation at 0Δtime and 1Δtime. Macro diffusion coefficient was estimated from the slope of the fitted line through the entire MSD vs. time plot. A statistical variance model was used to calculate domain size and resident time as previously described (50). In brief, a sliding window analysis was performed to determine the normalized variance in the position of the receptor within the time windows. Windows were then translated alongside of particle trajectories. The time of the inter-domain jumps were indicated by peaks and collected as n + 1. The average diameter of an individual domain and resident time was calculated as previously described (50, 90, 91). A total of 122 and 104 receptor trajectories were

analyzed from cells collected from control and fish meal supplemented cows, respectively.

Plasma, Tissue, and Supplement Fatty Acid Analysis

Plasma (500 μL), luteal tissue (200 mg), or dietary supplement (100 mg) was added to a 16 × 100 mm reaction tube and freeze-dried for 24 h prior to methylation. Samples were covered and maintained in the dark to minimize light-induced oxidation of fatty acids. Fatty acids were methylated using direct methylation as previous described (72). Long-chain fatty acid composition was determined using an Agilent 7890A Series GLC (Wilmington, DE) with a MS detector. The instrument was equipped with a 30-m × 0.20-mm (i.d.) fused silica capillary column (Supelcowax10; Supelco Inc., Bellefonte, PA, USA). The fatty acid methyl ester preparations were injected (1 μL) using the splitless mode. The carrier gas was helium, and the oven temperature was programmed from an initial temperature of 140°C that was held for 10 min and then increased to a final temperature of 250°C at the rate of 2.5°C/min. The final temperature was held for 10 min for a total run time of 65 min. Chromatograms were generated with a computing integrator (ChemStation Plus Chromatograph Manager; Agilent Technologies, Boulder, CO, USA). Standard fatty acid methyl ester mixtures were used to calibrate the GLC system using reference standard GLC 68-D (Nu-Chek Prep, Inc. Waterville, MN, USA). Palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) α-linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6) were identified by comparing the mass spectrometry analysis and relative retention times of fatty acid methyl ester peaks of samples with

those of standards. These peaks were then calculated as normalized area percentages of fatty acids.

Statistical Analysis

All data are reported as least square means ± standard error of the mean and significance was declared at $P < 0.05$. Effects of dietary supplementation on initial and ending BW, luteal fatty acid composition, lipid microdomain staining intensity, lateral mobility of FP receptors (micro and macro diffusion coefficients), domain sizes and resident time of receptors were analyzed using 1-way analysis of variance. The model included dietary supplementation, cow, and residual error as sources of variation. Cow was considered a random variable in the model. Calculations were made using the mixed model procedure of SAS and means were compared by *t*-tests using the PDIFF option of SAS. The effects of dietary supplementation on plasma fatty acid content was analyzed using 1-way analysis of variance with repeated measures. The statistical model included dietary supplementation, day, cow, supplementation × day, and residual error as sources of variation. Cow was considered a random variable in the model. Calculations were made in SAS using mixed modeling procedure and the repeated statement. A heterogeneous autoregressive covariance structure was used in the repeated model to account for heterogeneous variation among samples. Pre-planned pairwise *t*-test comparisons were used to determine differences using the PDIFF option of SAS.

Results

Changes in Body Weight

Initial BW was 463 ± 11.3 kg for control cows and 478 ± 11.3 kg for fish meal animals and did not differ ($P > 0.05$). All cows gained BW during the supplemental

period. Likewise, ending body weight did not differ ($P > 0.05$) between supplementation groups and was 515 ± 18.1 kg for control cows and 521 ± 18.1 kg for fish meal supplemented animals.

Plasma and Luteal Fatty Acid Composition

There was no effect of dietary supplementation, day, or dietary supplementation × day on plasma fatty acid content of palmitic, palmitoleic, stearic, oleic or arachidonic acids ($P > 0.10$; data not shown). Plasma linoleic acid was greater in cows receiving control treatment as compared to cows receiving treatment supplement ($P < 0.05$; data not shown). The effects of dietary supplementation on plasma omega-3 fatty acid composition during the supplemental period are shown in Figure 44. There was no effect of dietary supplementation, day, or dietary supplementation × day interaction on plasma α-linolenic ($P > 0.05$). However, there was an effect of dietary supplementation, day, and dietary supplementation × day interaction on plasma eicosapentaenoic and docosahexaenoic acids ($P < 0.05$). Plasma eicosapentaenoic or docosahexaenoic acid did not differ ($P > 0.10$) between controls or fish meal supplemented cows at the beginning of the experiment (d 0). However, cows supplemented with fish meal had higher plasma eicosapentaenoic acid starting at d 28 and docosahexaenoic acid starting at d 21 and both remained higher for the remainder of the supplemental period ($P < 0.05$).

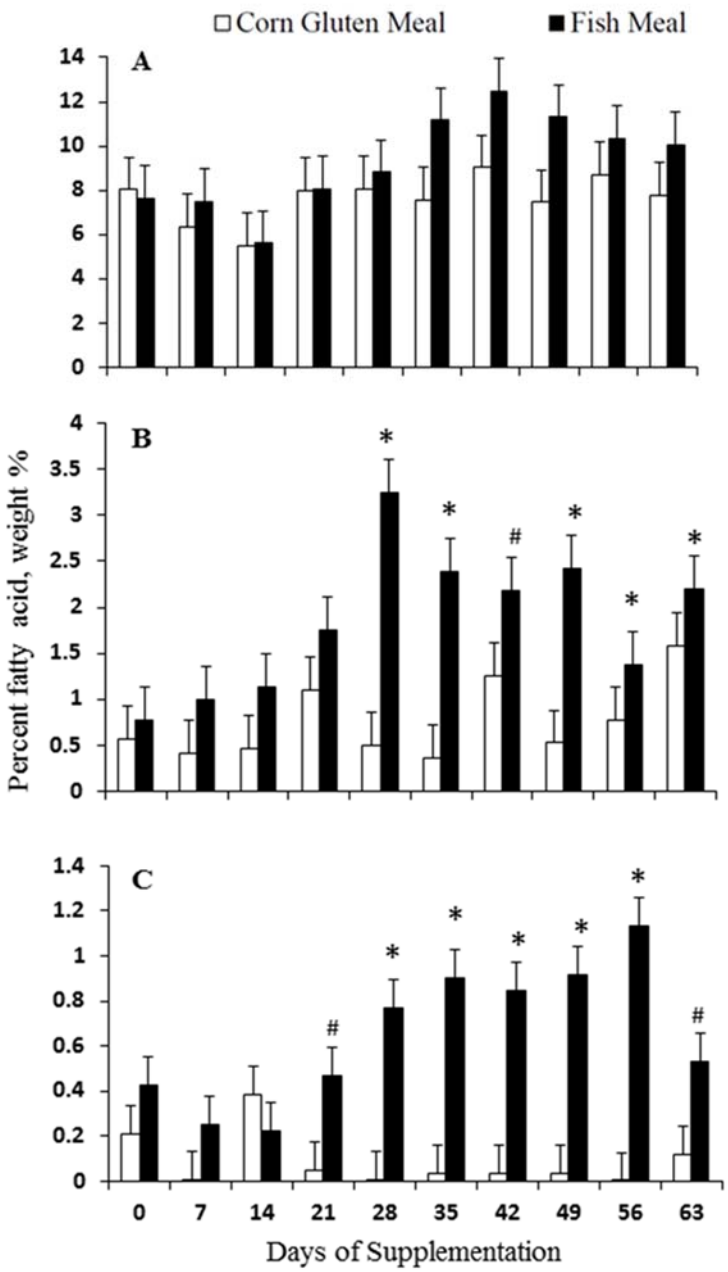


Figure 44. Effects of dietary supplementation on relative composition of plasma omega-3 fatty acids. Panel A: α-Linolenic acid; dietary supplementation ($P > 0.05$), day ($P > 0.05$), and dietary supplementation × day interaction ($P > 0.05$). Panel B: Eicosapentaenoic acid; dietary supplementation ($P < 0.05$), day ($P < 0.05$) and dietary supplementation × day interaction ($P < 0.05$). Panel C: Docosahexaenoic acid; dietary supplementation ($P < 0.05$), Day ($P < 0.05$) and dietary supplementation × day interaction ($P < 0.05$). #Significance differences within day of supplementation, $P < 0.05$. *Significance differences within day of supplementation, $P < 0.001$.

The effects of dietary supplementation on luteal fatty acid composition are shown in Figure 45. There was no effect of dietary supplementation on luteal content of palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic acids ($P > 0.05$). However, luteal content of α-linolenic, eicosapentaenoic, and docosahexaenoic acids were greater in tissue obtained from fish meal supplemented cows as compared to control cows ($P < 0.05$).

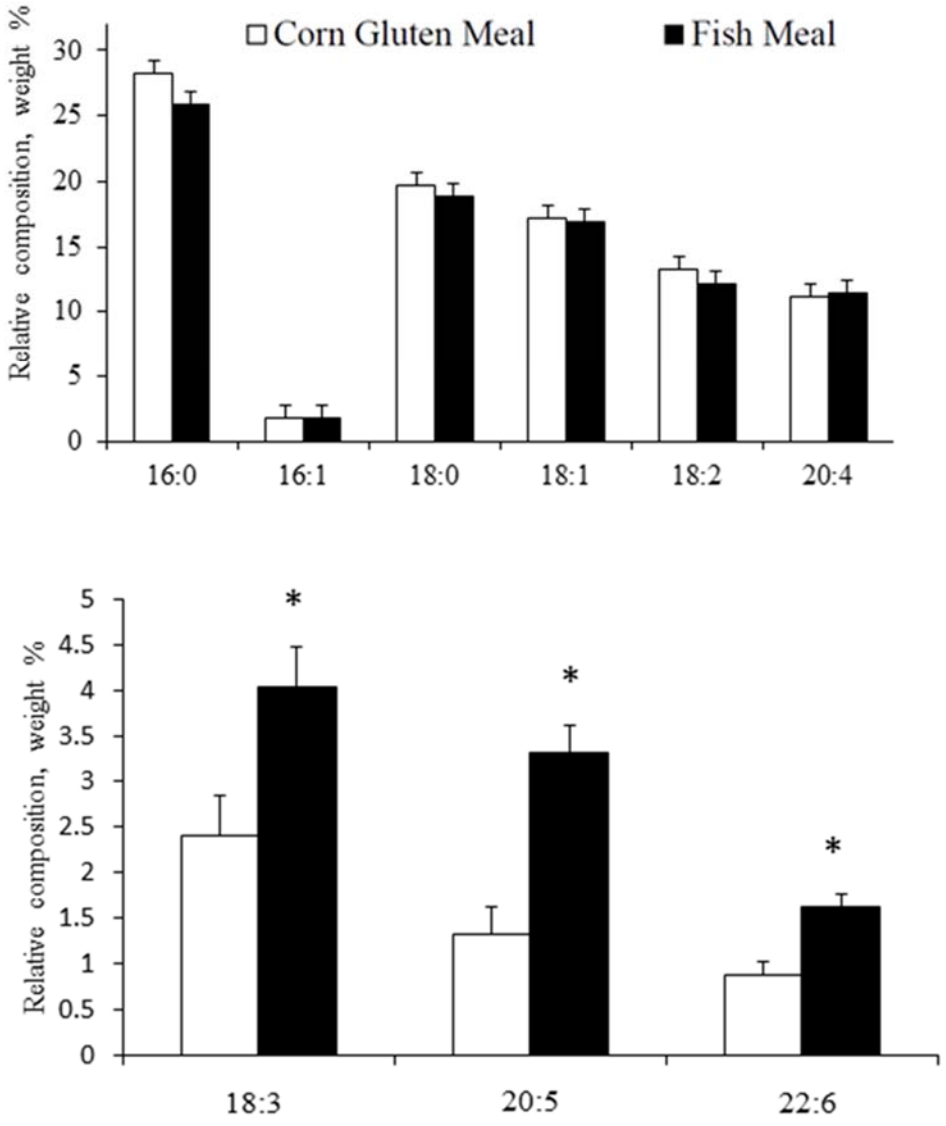


Figure 45. Effects of dietary supplementation on relative composition of luteal long-chain fatty acids. Panel A: Relative composition (weight percent) of palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4). Panel B: Relative composition (weight percent) of luteal omega-3 fatty acids α-linolenic acid (18:3), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6). *Significant difference within class of fatty acid; $P < 0.05$. Corn gluten meal supplemented animals (n = 4; open bars); fish meal supplemented animals (n = 4; solid bars).

Spatial Distribution of Lipid Microdomains in Dissociated Luteal Cells

Antibody cross-linking of monosialotetrahexosylganglioside (GM_1) resulted in formation of distinct patches on the plasma membrane of live, non-fixed dissociated cells. Representative cells from tissue acquired from a control and a fish meal supplemented cow are shown in Figure 46. The spatial distribution of microdomains of cells obtained from fish meal supplemented cows were disrupted resulting in a 52% decrease in total cell fluorescent intensity as compared to cells obtained from controls (Fig. 46; $P < 0.05$). Cells were also treated with β-MCD to examine the effects of cholesterol on lipid microdomain structure in these cells. Regardless of source of tissue (control or fish meal supplemented animals), depletion of cholesterol by β-MCD resulted in reduced total cell fluorescent intensity when compared to untreated cells ($P < 0.05$). Cells from control animals that were depleted of cholesterol with β-MCD resulted in a 62% decrease in total cell fluorescent intensity; cells from fish meal supplemented cows that were depleted of cholesterol had a 56% reduction in total cell intensity ($P < 0.05$).

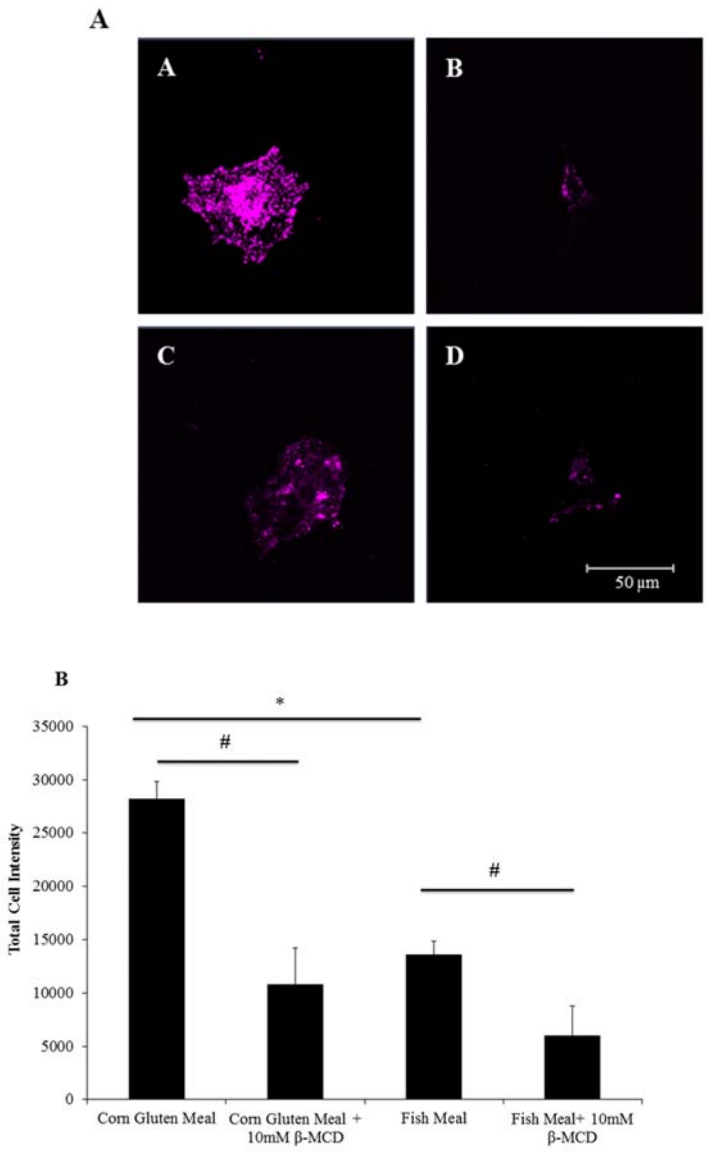
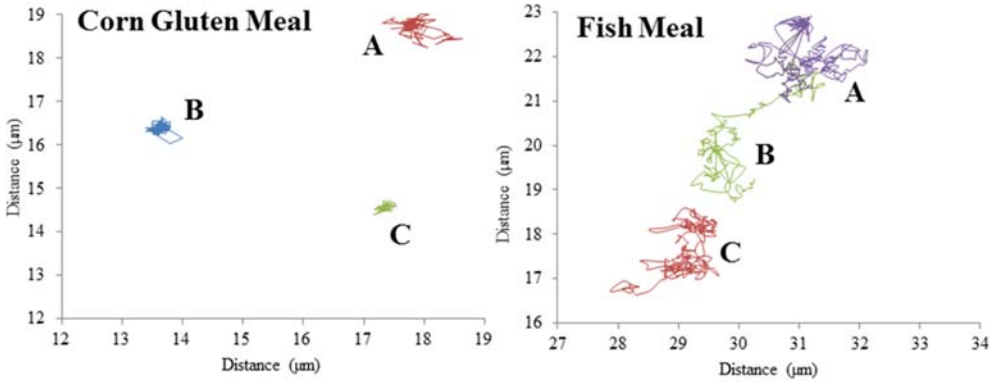


Figure 46. Effects of dietary supplementation on lipid microdomain structure on the plasma membrane of dissociated bovine luteal cells. Panel A: Representative cell obtained from (A) corn gluten meal supplemented animal, (B) individual cell obtained from same corpus luteum of corn gluten meal supplemented animal and subsequently treated with 10 mM beta-methylcyclodextrin (β-MCD), (C) individual cell obtained from fish meal supplemented cow and, (D) individual cell obtained from same corpus luteum of fish meal supplemented animal and subsequently treated with β-MCD. Panel B: Mean total cell intensity for cells obtained from corn gluten meal cows (n = 4; n = 366 cells), cells from corn gluten meal cows and treated with β-MCD (n = 80 cells), cells from fish meal supplemented animals (n = 4 cows; n = 578 cells), and cells from fish meal supplemented animals and treated with β-MCD (n = 117 cells). *$P < 0.05$; corn gluten meal vs. fish meal; #$P < 0.05$; corn gluten meal vs. corn gluten meal treated with β-MCD, and fish meal vs. fish meal treated with β-MCD.

Lateral Mobility of FP Receptors on Dissociated Luteal Cells

Representative FP receptor trajectories of cells from CL obtained from control and fish meal supplemented cows are shown in Figure 47. There was an effect of dietary supplementation on both micro and macro diffusion coefficients. Micro diffusion of FP receptors on cells obtained from fish meal supplemented animals was increased by 152% as compared to receptors on cells from controls (Fig. 48; $P < 0.05$). Furthermore, macro diffusion was increased by 178% in cells obtained from fish meal supplemented animals (Fig. 48; $P < 0.05$).



Effects of fish meal on lateral mobility of prostaglandin $F_{2\alpha}$ receptors in bovine luteal cells.

	Diffusion Coefficient		Domain Characteristics	
Treatment	D_{micro} (µm²/s)	D_{macro} (µm²/s)	Residence Time(s)	Domain Size (µm)
Corn Gluten Meal A	0.106	0.00002	4.79	0.019
Corn Gluten Meal B	0.111	0.00002	7.26	0.024
Corn Gluten Meal C	0.040	0.00002	4.78	0.019
Fish Meal A	0.638	0.002	4.25	0.154
Fish Meal B	0.743	0.033	2.78	0.607
Fish Meal C	0.608	0.010	4.42	0.433

Figure 47. Prostaglandin F_{2alpha} receptor trajectories. Three individual FP receptor trajectories (A,B,C) on cell membranes obtained from a corn gluten meal supplemented cow and fish meal supplemented cow. The table shows calculated micro diffusion coefficient (D_{micro}), macro diffusion coefficient (D_{macro}), domain size, and residence time for each individual receptor.

Dietary supplementation affected diameter, also referred to as corals, of lipid microdomains. Domains associated with FP receptors were 2–fold larger for cells obtained from fish meal supplemented animals as compared to cells obtained from controls (Fig. 48; $P < 0.05$). Dietary supplementation also affected the time a receptor resided within a domain. Receptors of cells from CL obtained from cows supplemented with fish meal were retained for 22% less time in a domain as compared to receptors of cells from tissue obtained from controls (Fig. 48; $P < 0.05$).

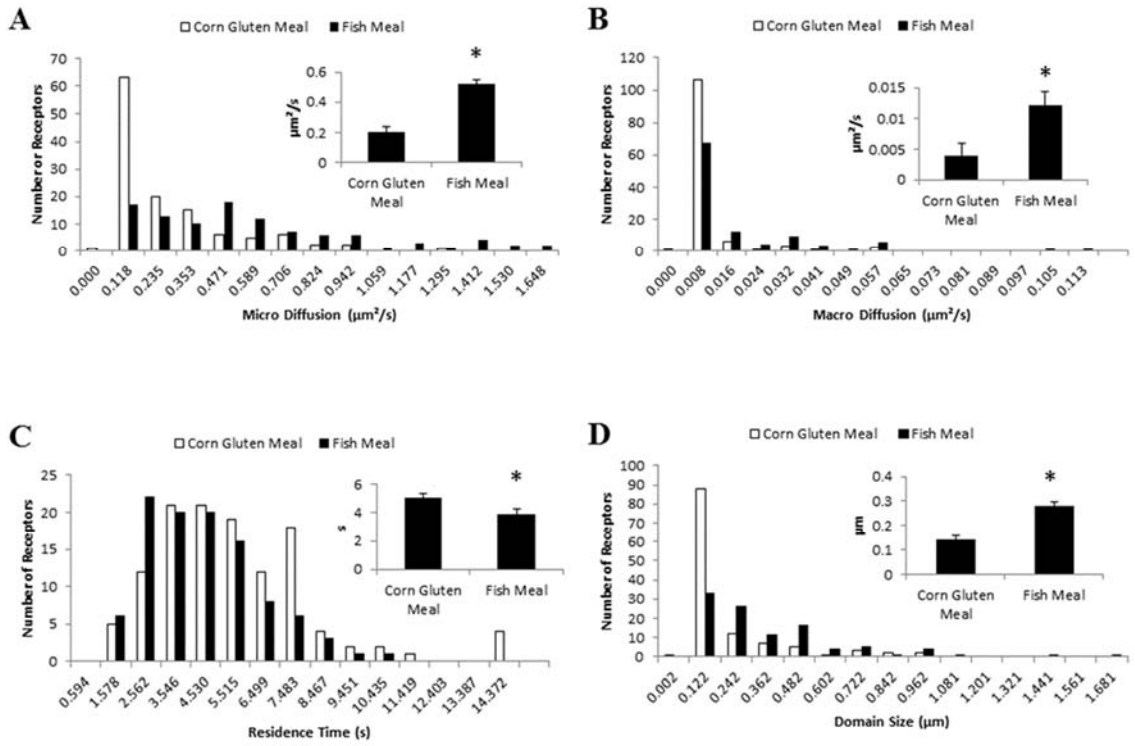


Figure 48. Effects of dietary supplementation on lateral mobility of the prostaglandin F_{2alpha} receptor. Distribution of micro diffusion coefficient (A), macro diffusion coefficient (B), residence time (C), and microdomain diameter (D) of FP receptors in luteal tissue collected from corn gluten meal supplemented cows (n = 4; open bar; n = 122 receptors) or fish meal supplemented cows (n = 4; solid bar; n = 104 receptors). Diffusion coefficients were calculated using mean square displacement and domain size using sliding window variance (see Material and Methods). The inset in each panel shows the mean ± standard error of the mean (* $P < 0.05$).

Discussion

The present study was undertaken to examine the influence of dietary supplementation of fish meal on lipid microdomain structure and lateral mobility of FP receptors obtained from dissociated cells of the bovine CL. Inclusion of rumen-grade menhaden fish meal in the diet of beef and lactating dairy cows has been reported to improve pregnancy rates (198, 200, 217). However, the mechanism by which fish meal improves pregnancy is still largely unknown. Many studies have been conducted that examine influences of fish meal on uterine prostaglandin metabolism (40, 43, 88), but little work regarding CL function has been conducted. Menhaden fish meal contains approximately 11% crude fat with a high percentage of long-chain polyunsaturated omega-3 fatty acids, eicosapentaenoic and docosahexaenoic acid (Table 2). This class of fatty acids has been shown to affect lipid microdomain structure (53, 218) and lateral mobility of membrane bound receptors (50). However, to our knowledge, the influence of these fatty acids on lipid microdomains or receptor mobility have not be investigated with cells of the bovine CL *in vivo.*

Cows supplemented with fish meal had increased plasma and luteal eicosapentaenoic and docosahexaenoic fatty acids which is in agreement with previous studies from this (43, 69, 72) and other laboratories (219). Nutrition, especially energy balance, can have a significant impact on follicular growth and subsequent CL function in beef and dairy cows (220-222). However, cows in this study had similar gain in BW during the supplementation period and differences in plasma and luteal omega-3 fatty acid composition were due to changes in dietary fatty acids received from the supplementation and not attributed to plane of nutrition.

One of the objectives of this study was to examine the effects of dietary fish meal supplementation on lipid microdomain structure of dissociated cells of the CL. Lipid microdomains are microscopic regions ranging in size from 10 to 200 nm in diameter that are rich with cholesterol and sphingolipids, primarily sphingomyelin and GM_1 (210). Binding of cholera toxin to GM_1 followed by cross-linking with antibodies is a common approach that can be used to visualize microdomains on plasma membranes of cells (157). In this study using live, non-fixed dissociated cells of the CL, lipid microdomains were detected as punctated patches on membranes from cells collected from both supplemented groups. Spatial distribution of microdomains on membranes of cells from tissue collected from fish meal supplemented animals were more dispersed (i.e., less clustering of GM_1 on the plasma membrane) resulting in decreased total cell fluorescent intensity. Similar effects of omega-3 fatty acids on lipid microdomain structure have been reported for cells of the CL (50) and immune cells (53, 150, 223) treated with fish oil. Cholesterol also plays an important role in lipid microdomain integrity (224, 225). Removal of cholesterol by culturing cells in the presence of β-MCD resulted in dispersion of these domains in cells from tissue collected from controls, which is in agreement with other studies (50, 224). Likewise, cells obtained from fish meal supplemented cows, cultured in the presence of □-MCD, resulted in further disruption of microdomains. A limitation of this study was use of dissociated cells. The CL is glandular tissue composed of many cell types which include steroidogenic (large and small), endothelial, fibroblasts, pericytes, and immune (201-203). Therefore, it was not possible to determine the specific cell type that was influenced by dietary supplementation in the current study. However, it was apparent that most all cells

collected from fish meal supplemented animals had considerable disruption of lipid microdomains. Enrichment of specific populations of cell types of the CL and effects of omega-3 fatty acids on lipid microdomain structure is warranted in future studies.

A second objective of this study was to investigate the influence of fish meal supplementation on lateral mobility of FP receptors using single particle tracking. Micro and macro diffusion coefficients, domain size, and resident time of receptors on FP positive cells obtained from controls were within values reported in the literature for membrane-bound receptors using single particle tracking methodology (214, 226-232). Here in, we show that altered mobility of FP receptors on the plasma membrane was associated with an increase in omega-3 fatty acids in bovine luteal tissue. Lateral mobility of the FP receptor, as determined by micro and macro diffusion, was increased in FP positive cells of tissue collected from cows supplemented with fish meal. Furthermore, the average time each receptor resided in a domain was decreased compared to receptors on cells obtained from controls. Likewise, increased domain sizes were observed in cells collected from fish meal supplemented animals which is consistent with a recent *in vitro* study from this laboratory showing fish oil increased domain size (50). In the cow, FP receptors are expressed on the plasma membrane of steroidogenic (both large and small) (24, 138) and endothelial cells (206, 233). Therefore, it was not possible to identify the specific cell type that expressed FP receptors in the current study and warrants additional investigation.

Results from this study clearly demonstrate that fish meal supplementation increases luteal omega-3 fatty acid content in bovine CL and has a dramatic impact on lipid microdomain structure and FP receptor mobility. The mechanisms by which these

fatty acids alter membrane dynamics and lipid microdomain structure are largely unknown. As opposed to bulk lipids of the plasma membrane, a characteristic of sphingolipids in lipid microdomains is the increased unsaturated fatty acyl chains giving rise to a more quasi-liquid ordered state. However, glycerophospholipids within the plasma membrane have unsaturated or polyunsaturated fatty acid acyl chains and are loosely packed together in a quasi-liquid disordered fashion giving the membrane its fluid property (234-236). Therefore, one mechanism is that eicosapentaenoic and docosahexaenoic fatty acids from the dietary marine oils become esterified to glycerophospholipids and increase the quasi-liquid disorder of the plasma membrane, which in turn increases fluidity of the membrane and lateral mobility of the FP receptor.

Another possible mechanism is that incorporation of long-chain polyunsaturated fatty acids into lipid microdomains of biological membranes potentially alters protein distribution, protein-lipid association, and/or protein-protein interactions. Reports in the literature show polyunsaturated long-chain fatty acids including eicosapentaenoic and docosahexaenoic acids can be incorporated into sphingolipids and thereby influence the quasi-liquid state of the membrane lipid microdomain from ordered to more disorder (53, 218). This disruption of quasi-liquid ordered state may displace membrane receptors from lipid microdomains. Single particle tracking data from the current study show a reduction in resident times, supporting this hypothesis.

Maintaining adequate progesterone secretion by the CL following mating is essential for successful pregnancy (8, 237-239). In the bovine, the conceptus must adequately control uterine prostaglandin secretion allowing for continued synthesis of progesterone from the CL. Interferon tau is the signal produced by the trophoblastic cells

of the developing conceptus that alters uterine prostaglandin synthesis, thereby allowing for the establishment of pregnancy (209). The window in which the conceptus has to produce an adequate amount of interferon tau is crucial for regulation of $PGF_{2\alpha}$. The trophoblastic cells of slowly developing embryos have been reported to secrete insufficient interferon tau or have delayed release (240). Regression of the gland and loss of the pregnancy is a direct result of failing to inhibit the secretion of $PGF_{2\alpha}$ from the uterus. Inclusion of fish meal into the diet of breeding females may be one way to regulate uterine $PGF_{2\alpha}$ secretion or luteal sensitivity.

Recent *in vitro* and whole animal studies support the hypothesis that fish by-products alter luteal sensitivity to $PGF_{2\square}$. We have demonstrated that treating cells of the CL *in vitro* with fish oil increased lateral mobility of FP receptors and decreased confinement within the lipid microdomains. Moreover, addition of $PGF_{2\square}$ did not influence mobility or confinement of receptors in fish oil treated cells, while $PGF_{2\square}$ decreased mobility and increased confinement of receptors in control cells (50). In addition, we have observed that intrauterine infusion of low doses of $PGF_{2\square}$ between d 10 to 12 of the estrous cycle resulted in 56% less CL undergoing functional regression in fish meal supplemented cows when compared to controls (unpublished data). Taken together, these data and the current study show that incorporation of omega-3 long-chain polyunsaturated fatty acids from fish by-products can affect mobility of integral proteins associated with the plasma membrane. This change in membrane dynamics can influence the topology of the receptors and subsequent sensitivity to ligand. Therefore, a reduction in sensitivity to $PGF_{2\alpha}$ may increase the window for trophoblastic cells to secrete necessary amount of interferon tau, thus improving reproductive success in the bovine.

In conclusion, inclusion of fish meal in the diet of cows affected CL membrane lipid microdomains and mobility of FP receptors, likely impacting membrane fluidity and microdomain structure. Alteration of lipid microdomain structure and mobility of FP receptors may reduce $PGF_{2\alpha}$ signaling in the bovine CL. Reduction in $PGF_{2\alpha}$ signaling within the CL at the time of maternal recognition of pregnancy may improve pregnancy outcome in times when a slowly developing embryo does not adequately regulate uterine $PGF_{2\alpha}$ secretion.

CHAPTER 7

EFFECT OF FISH MEAL SUPPLEMENTATION ON LUTEAL SENSITIVITY TO INTRAUTERINE INFUSIONS OF PROSTAGLANDIN F2ALPHA IN THE BOVINE

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Contribution of Authors and Co-Authors

Manuscript in Chapter VII

Author: Michele R. Plewes

Contributions: Developed and implemented the study design. Generated and analyzed data. Wrote first draft of the manuscript.

Co-Author: Patrick. D. Burns

Contributions: Helped conceive the study topic. Provided guidance on study design. Provided feedback data interpretation and manuscript preparation.

Co-Author: Peter. E Graham

Contributions: Helped conceive the study topic. Provided guidance on study design.

Co-Author: Jason Bruemmer

Contributions: Helped conceive the study topic. Provided guidance on study design.

Co-Author: Terry Engle

Contributions: Helped conceive the study topic. Provided guidance on study design.

Abstract

Progesterone is a steroid secreted from the corpus luteum (CL), which is responsible for establishment and maintenance of pregnancy. In non-pregnant ruminants, prostaglandin (PG) F_{2alpha} is released from the uterus causing regression of the CL and another opportunity for mating. Early embryonic mortality often occurs due to inadequate regulation of uterine PGF_{2alpha} secretion, leading to a decrease in progesterone and loss of pregnancy. The objective of the current study was to determine the effects of fish meal supplementation on luteal sensitivity to intrauterine infusions of PGF_{2alpha}. Non-lactating beef cows receive corn gluten meal (n = 17) or fish meal (n = 16) supplementation for 60-days. Cows were administered four intrauterine infusions of 0.25 mL saline at 6-h intervals (n = 6 corn gluten meal; n = 5 fish meal) or 2 doses of 0.5 mg PGF_{2alpha} in 0.25 mL saline at 12-h intervals (n = 11 corn gluten meal; n = 11 fish meal) commencing on day 10 to 12 of the cycle. Trans-rectal ultrasonography was performed to measure diameter of CL at time of each uterine infusion and at 48 h. Blood samples were collected immediately before infusion and at 3-h intervals for 24 h and then 6-h intervals for an additional 24 h to measure serum progesterone. Intrauterine infusion of PGF_{2alpha} resulted in functional regression for 90% of cows supplemented with corn gluten meal, and only 46% for animals supplemented with fish meal. Results demonstrate fish meal supplementation decreases luteal sensitivity to PGF_{2alpha}, which may improve fertility.

Introduction

The corpus luteum (CL) is an ovarian endocrine gland that is essential for the maintenance and establishment of pregnancy in all mammalian females. In the cow, a surge of luteinizing hormone (LH) is released from the anterior pituitary gland after onset

of estrus to cause follicular rupture and release of the ovum (241-243). In addition, LH causes the theca and granulosa cells of the ovulated follicle to differentiate in small and large steroidogenic luteal cells, respectively (5, 202, 244). Working as a biological clock regulating the estrous cycle, the steroidogenic cells of the CL synthesize and secrete progesterone. Progesterone release from the CL has many functions which include blocking estrogen-induced pituitary release of gonadotrophins (9) and embryo development (31, 32).

In the bovine, prostaglandin (PG) $F_{2\alpha}$ is released by the uterus in the event that fertilization has not occurred, causing regression of the CL (136, 160, 205). In the non-pregnant cow, $PGF_{2\alpha}$ binds to the prostaglandin $F_{2\alpha}$ (FP) receptor, initiating an intracellular signaling cascade, leading to inhibition of progesterone synthesis and regression of the gland (15, 26). In the pregnant cow, the embryo must produce sufficient amounts of interferon-τ to trigger maternal recognition of pregnancy (1, 132, 209). Interferon-τ secreted from the trophectoderm of the developing conceptus attenuates uterine release of $PGF_{2\alpha}$ preventing regression of the gland. Early embryonic mortality often occurs when a viable embryo fails to effectively control $PGF_{2\alpha}$ secretion, resulting in regression of the CL and termination of pregnancy (10). Embryos failing to inhibit $PGF_{2\alpha}$ luteolytic activity is a major cause of pregnancy loss in bovine (132, 143). Previous results from our laboratory demonstrated supplementation of fish meal increases the lateral mobility of the bovine FP receptors (153), which may decrease luteal sensitivity to $PGF_{2\alpha}$.

Nutrition, especially energy, plays a critical role on reproductive performance in the bovine (33-37). The omega-3 polyunsaturated fatty acids are a distinct class of long-

chain fatty acids with a double bond at the third carbon from the methyl end of the acyl chain. There are three omega-3 fatty acids that have a significant impact on cellular physiology and possibly reproduction: α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Numerous studies have been conducted showing beneficial effects of omega-3 polyunsaturated fatty acids on ovarian (38, 39) and uterine function in the bovine (40, 41). The mechanism by which a diet high in omega-3 polyunsaturated fatty acids alter reproductive performance is still largely unknown.

Supplementation of fats in the diet, specifically sources high in omega-3 polyunsaturated fatty acids, have been shown to influence ovarian function, steroidogenesis, and gene expression (245). Additionally, supplementation of fats having a high percentage of omega-3 polyunsaturated fatty acids has been reported to suppress oxytocin-induced uterine synthesis of $PGF_{2\alpha}$ cattle, which may contribute to a reduction in embryonic mortality (42, 43). Therefore, supplementing the diet of breeding females with fish meal, a rich source of omega-3 polyunsaturated fatty acids, may regulate uterine $PGF_{2\alpha}$ secretion. However, these fatty acids may alter luteal sensitivity to $PGF_{2\alpha}$ as well.

We have reported that dietary supplementation of fish meal influences $PGF_{2\alpha}$ receptor mobility (153). Increased mobility of FP receptors may reduce luteal sensitivity to $PGF_{2\alpha}$, thereby, increasing the window for viable embryos to trigger maternal recognition of pregnancy. Therefore, it is hypothesized that fish meal supplementation may lead to a decrease in luteal sensitivity to $PGF_{2\alpha}$ in the bovine. The objects of this study were to determine the effects of dietary fish meal supplementation on 1) luteal diameter, and 2) circulating serum progesterone concentrations in response to intrauterine infusions of $PGF_{2\alpha}$.

Methods

Animals, Diets, and Blood Sampling

All animal procedures described herein were approved by the Colorado State University institutional animal care and use committee (Approval # 13 - 4440A). Beef cows of mixed breeds were purchased at a local sale barn in Fort Collins, Colorado and housed in dry lots at the Colorado State University Animal Reproduction Biotechnology Laboratory Foothills campus. Reproductive organs were palpated per rectum for presence of gross anatomical abnormalities (cystic follicles) and adhesions. In addition, trans-rectal ultrasonography using an Aloka 500 V equipped with a 5 MHz linear array transducer was performed on ovaries for presence of CL and uteri for absence of a fetus. Cows with adhesions, cystic follicles, absence of a CL, or pregnant were removed from the study.

Cows received a 95% mixed hay diet (Table 5) throughout the supplementation period. Cows were stratified by BW and randomly assigned to receive corn gluten meal (n = 17) or fish meal (n = 16; SeaLac, Omega Protein; FIG 49) supplementation. Diets were delivered daily to cows for approximately 60-days, at a dry matter intake equivalent to 2.0% BW (Table 6). Supplements were formulated to be isocaloric and isonitrogenous, meeting or exceeding NRC recommendations for non-lactating beef cows (153). Animals were individually penned twice daily at 0600 and 1800 daily for approximately 2 to 3 h to receive hay and supplement. After which, animals were released from pens to have *ad libitum* access to shelter and water. Body weights were collected weekly to monitor changes in BW, and diets were adjust as needed to maintain desired supplementation at 5% dry matter intake.

Table 5. Chemical composition of mixed grass hay

Dry matter intake, %	95
Chemical Analysis	
Water Soluble Carbohydrates, %	8.7
Neutral Detergent Fiber, %	56.7
Acid Detergent Fiber, %	37.0
Simple Sugars, %	5.3
Starch, %	1.0
Non Fiber Carbohydrates, %	1.0
Crude Protein, %	15.2
Crude Fat, %	3.5

Table 6. Ingredient, chemical composition, and long-chain fatty acid profile of dietary supplementation

Item	Experimental Diet	
	Corn Gluten Meal	Fish Meal
Dry Matter Intake, %	5	5
Ingredient of Pelleted Supplement, %		
Fish meal	0	60.0
Corn Gluten meal	59.3	0
Wheat midds	19.2	17.4
Wheat – ground	7.3	7.8
Limestone	7.4	7.4
Molasses cane	4.0	4.0
Salt	1.3	1.3
Soybean oil-mixer	0.25	0.8
Magox	0.3	0.3
Monocal 16 21	0.25	0.25
Z_nSO_4	0.2	0.2
Se	0.1	0.1
M_nSO_4	0.1	0.1
$CuSO_4$	0.1	0.1
Vitamin A 30/3	0.1	0.1
Vitamin E 125	0.02	0.02
Vitamin D 0/30	0.02	0.01
Ranch-o-dine	0.02	0.02
Chemical Analysis		
Crude Protein, %	39.8	40.1
Degradable Intake Protein, %	29.5	29.9
Undegradable Intake Protein, %	33.7	34.2
Total Digestible Nutrients, %	68.8	69.6
Crude Fat, %	3.0	3.5
Fatty Acid Composition of Supplement, wt %		
Palmitic Acid (16:0)	14.3	26.4
Palmitoleic Acid (16:1)	1.5	8.7
Stearic Acid (18:0)	17.8	4.6
Oleic Acid (18:1)	38.8	12.5
Linoleic Acid (18:2)	2.2	11.6
Alpha-Linolenic Acid (18:3)	2.2	2.5
Arachidonic Acid (20:4)	<0.5	1.1
Eicosapentaenoic Acid (20:5)	<0.5	9.0
Docosahexaenoic Acid (22:6)	<0.5	8.9

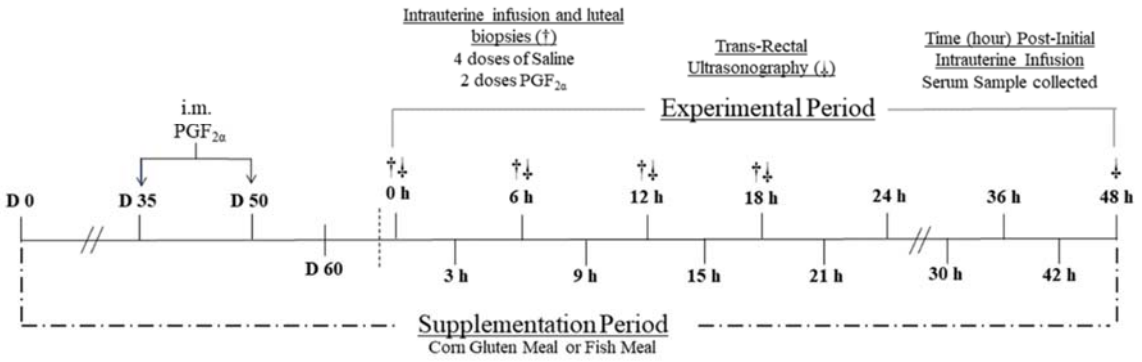

Figure 49. Schematic diagram of dietary supplementation and experimental design. Cows were supplemented corn gluten meal or fish meal and administered two injections of prostaglandin $F_{2\alpha}$ at 14-d intervals to synchronize estrous cycles. Cows were treated with intrauterine infusions of saline (4 doses at 6-h intervals) or $PGF_{2\alpha}$ (2 doses at 12-h intervals). Intrauterine Infusion (†), trans-rectal ultrasonography (⸸), and blood samples (h) were collected at indicated time points.

Jugular blood samples were collected immediately before supplementation commenced and weekly thereafter to measure changes in plasma fatty acid composition. Samples were collected in 3-mL blood tubes containing 5.4 mg EDTA (BD Vacutainer, Becton and Dickson Co) and immediately placed on ice. Samples were centrifuged at 1500 × *g* for 15 min, after which plasma was then collected and stored at -80°C until GLC analysis.

Cows were administered 25 mg i.m. injections of $PGF_{2\alpha}$ (Lutalyse, Pharmacia & Upjohn Co) on day 36 and 50 of supplementation to synchronize estrous cycles. Cows were observed for estrous behavior at dust and dawn for a minimum of 30 min following second injection of $PGF_{2\alpha}$. Heat detector patches (Estrotect) were applied to tail-head region of animal to aid in detection of estrus. Trans-rectal ultrasonography was performed on ovaries on day 10 to 12 post-estrus (approximately day 60 of the supplementation period) to confirm ovulation and presence of a CL (FIG 49).

Prostaglandin $F_{2\alpha}$ Intrauterine Infusions

Cows were administered four intrauterine infusions of 0.25 mL saline or 0.5 mg $PGF_{2\alpha}$ in 0.25 mL saline at mid-cycle (day 10 to 12 post-estrus). Animals were randomly assigned to receive either 4 doses of saline (controls; n = 6 corn gluten meal supplemented cows; n = 5 fish meal supplemented cows) at 6-h intervals or 2 doses of $PGF_{2\alpha}$ (n = 11 corn gluten meal supplemented cows; n = 11 fish meal supplemented cows) into the uterine horn ipsilateral to the CL. Cows administered 2 doses of $PGF_{2\alpha}$ were infused with $PGF_{2\alpha}$ at the first (0 h) and third (12 h) infusion and saline at the second and forth infusion (FIG 49). Using sterile techniques, 0.25 cc polyvinyl AI straws were pre-loaded with appropriate treatment immediately before administration.

Treatments were delivered at the curvature of the uterine horn ipsilateral to the ovary bearing the CL using an AI stylet by a skilled technician.

Corpus Luteum Diameter

Real-time trans-rectal ultrasonography was performed on the ovary bearing the CL using an Aloka 500 V equipped with a 5 MHz linear array transducer. Ovaries were scanned at the time of each uterine infusion and an additional scan 48 h following initial infusion. Diameter of CL was determined using digital calipers and recorded to the nearest 0.5 mm.

Progesterone Analysis

Jugular blood samples were collected immediately before infusion and at 3-h intervals for the first 24 h. After which, samples were collected at 6-h intervals for an additional 24 h (FIG 49). Blood samples were allowed to clot at 4 °C and were centrifuged at 1500 × g for 20 min. Serum was harvested and stored at -20 °C until assayed for progesterone.

A commercially available progesterone competitive ELISA assay (Cayman Chemical) was used to measure serum progesterone according to manufacturer's protocol. Progesterone was extracted using a double-extraction procedure prior to ELISA assay as described (246). In brief, 100 µL of serum and 1 mL petroleum ether were mixed in a 10 × 13 mm glass test tube. Phase separation was accomplished by placing samples into a -80 °C freezer for 5 min. The organic phase was decanted into a clean glass test tube and an additional 1 mL petroleum ether was added to aqueous phase and phases were separated as above. Organic phases were combined and evaporated using N_2

gas. Samples were reconstituted in 1× ELISA Buffer at a 1:10 dilution. Intra- and inter-assay coefficient of variation was 7.2 and 16.3%, respectively, across 25 assays.

Gas-Liquid Chromatography

Plasma (500 μL) was added to a 16 × 100 mm glass reaction tube and freeze-dried for 24 h prior to methylation. To prevent light-induced oxidation of fatty acids, samples were covered and maintained in dark during freeze-drying process. Fatty acids were methylated using a direct methylation procedure as previously described (72). An Agilent 7890A Series GLC (Agilent) with a mass spectrometry detector was used to determine plasma long-chain fatty acid composition, as previously described (153). The instrument was calibrated using fatty acid methyl ester GLC 68-D (Nu-Chek Prep, Inc) as a reference standard. Unknown long-chain fatty acids in plasma were determined by comparing the mass spectrometry and relative retention times to the reference standard (palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, α-linolenic acid, arachidonic acid, EPA, and DHA). Identified peaks were then calculated as normalized area percentages of long-chain fatty acids. Long-chain fatty acids were reported as relative weight percent, which was calculated by dividing individual fatty acid area by total peak area within the chromatogram.

Statistical Analysis

All data are reported as least square means ± standard error of the mean and significance was declared at $P < 0.05$. The effects of dietary supplementation (corn gluten meal or fish meal) on initial and ending BW were analyzed using *t*-test. Calculations were made in SAS (Cary, North Carolina, USA) using PROC TTEST. The effects of dietary supplementation (corn gluten meal or fish meal) on plasma long-chain fatty acid content

were analyzed using one-way analysis of variance with repeated measures. The statistical model included dietary supplementation, day, cow, supplementation × day, and residual error as sources of variation. Cow was considered a random variable in the model. Calculations were made in SAS using PROC MIXED with the repeated statement. A heterogeneous autoregressive covariance structure was used in the repeated model to account for heterogeneous variation among samples. Pre-planned pairwise *t*-test comparisons were used to determine differences using the PDIFF option of SAS. Cows infused with 2 doses of $PGF_{2\alpha}$ were grouped as having a regressed or non-regressed CL at the end of the experimental period. A regressed CL was considered when serum progesterone was below 1 ng/mL for two consecutive sampling time points. The effects of dietary supplementation (corn gluten meal or fish meal) and treatment (saline, regressed $PGF_{2\alpha}$, or non-regressed $PGF_{2\alpha}$) on serum progesterone and CL diameter was analyzed using one-way analysis of variance with repeated measures. The statistical model included dietary supplementation, treatment, time, cow, supplementation × treatment × time, and residual error as sources of variation. Cow was considered a random variable in the model. Calculations were made in SAS using PROC MIXED and the repeated statement. A heterogeneous autoregressive covariance structure was used in the repeated model to account for heterogeneous variation among samples. Pre-planned pairwise *t*-test comparisons were used to determine differences using the PDIFF option of SAS. Percentage of regressed CL between supplement groups receiving 2 doses of $PGF_{2\alpha}$ was analyzed using chi-square. Calculations were made using procedure CATMOD of SAS.

Results

Changes in Body Weight

Initial BW was 510 ± 12.5 kg for corn gluten meal supplemented cows and 514 ± 12.5 kg for fish meal animals, which did not differ (P >0.05). All cows gained BW during the supplemental period; therefore, ending BW did not differ (P > 0.05) between supplementation groups, which was 529 ± 12.2 kg for corn gluten meal supplemented cows and 549 ± 12.2 kg for fish meal supplemented animals.

Plasma Fatty Acid Composition

There was no effect of dietary supplementation, day, or dietary supplementation × day on plasma fatty acid content of palmitic, palmitoleic, stearic, oleic or arachidonic acids (P > 0.10; data not shown). Plasma linoleic acid was greater in cows receiving corn gluten meal supplement as compared to cows receiving fish meal supplement (P < 0.05; data not shown). The effects of dietary supplementation on plasma omega-3 fatty acid composition during the supplemental period are shown in Figure 50. There was no main effect of dietary supplementation on plasma α-linolenic (P > 0.05). There was however, a dietary supplementation × day interaction with an increase of plasma α-linolenic acid on day 7, 21, and 35 for animals supplemented with fish meal (P < 0.05; FIG 50A). There was an effect of dietary supplementation, day, and dietary supplementation × day interaction on both plasma EPA and DHA (P < 0.05). Plasma EPA and DHA did not differ (P > 0.10) between corn gluten meal or fish meal supplemented cows at the beginning of the experiment (day 0). However, cows supplemented with fish meal had higher plasma EPA starting at day 7 and DHA at day 14. After which, plasma EPA and

DHA was greater for cows supplemented fish meal for the remainder of the supplemental period ($P < 0.05$; FIG 50B and 50C).

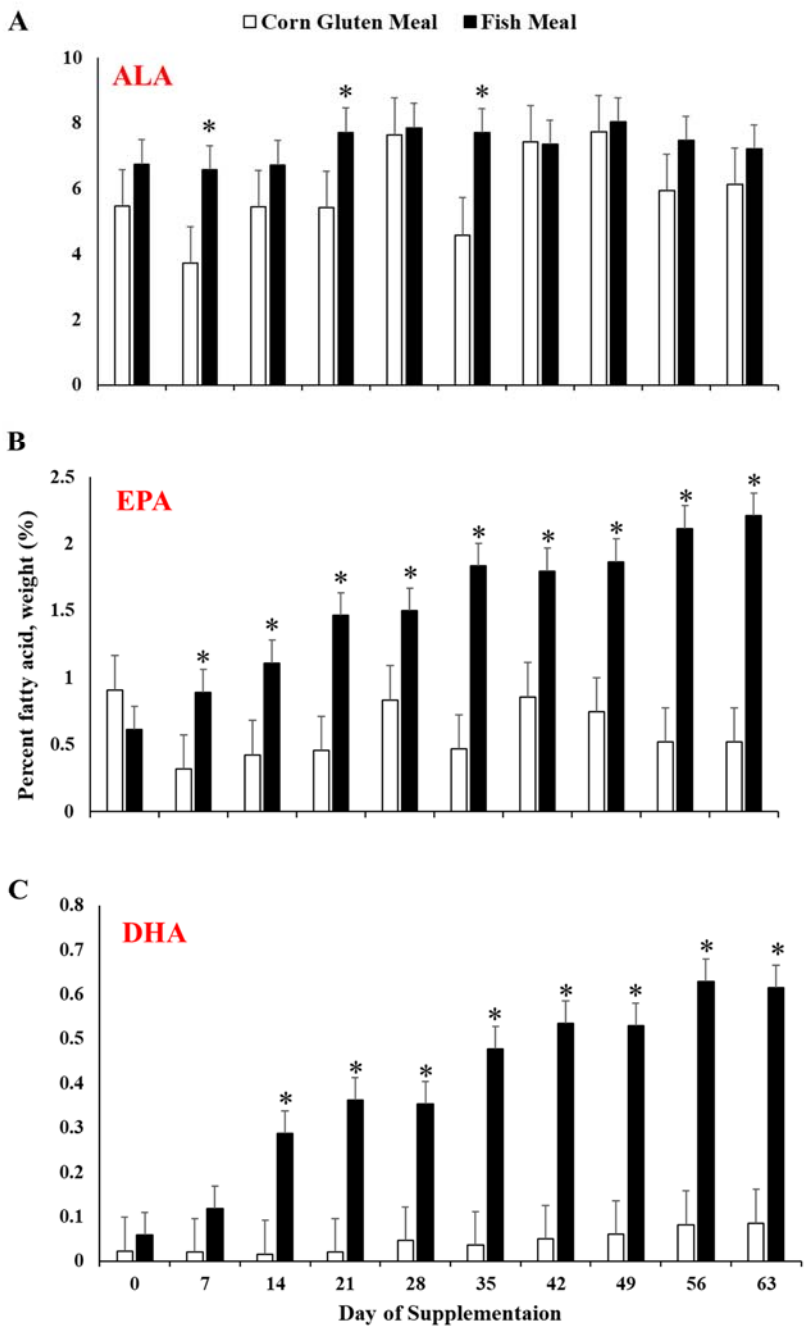

Figure 50. Effects of corn gluten meal or fish meal on relative composition of plasma omega-3 long-chain polyunsaturated fatty acids. Panel A: α-Linolenic acid; dietary supplementation ($P > 0.05$), day ($P > 0.05$), and dietary supplementation × day interaction ($P < 0.05$). Panel B: Eicosapentaenoic acid; dietary supplementation ($P < 0.05$), day ($P < 0.05$) and dietary supplementation × day interaction ($P < 0.05$). Panel C: Docosahexaenoic acid; dietary supplementation ($P < 0.05$), Day ($P < 0.05$) and dietary supplementation × day interaction ($P < 0.05$). *Significance differences within day of supplementation, $P < 0.05$.

Effects of Dietary Supplementation of Fish Meal on Serum Progesterone Following Intrauterine Infusions of $PGF_{2\alpha}$

In the current study, regression of the CL was defined as serum progesterone remaining below 1 ng/mL for two consecutive time points. There was no difference between dietary supplementation or treatment groups on initial progesterone concentrations (0 h; $P > 0.05$; FIG 51). Dietary supplementation did not influence serum progesterone concentrations in animals treated with 4 doses of saline ($P > 0.05$).

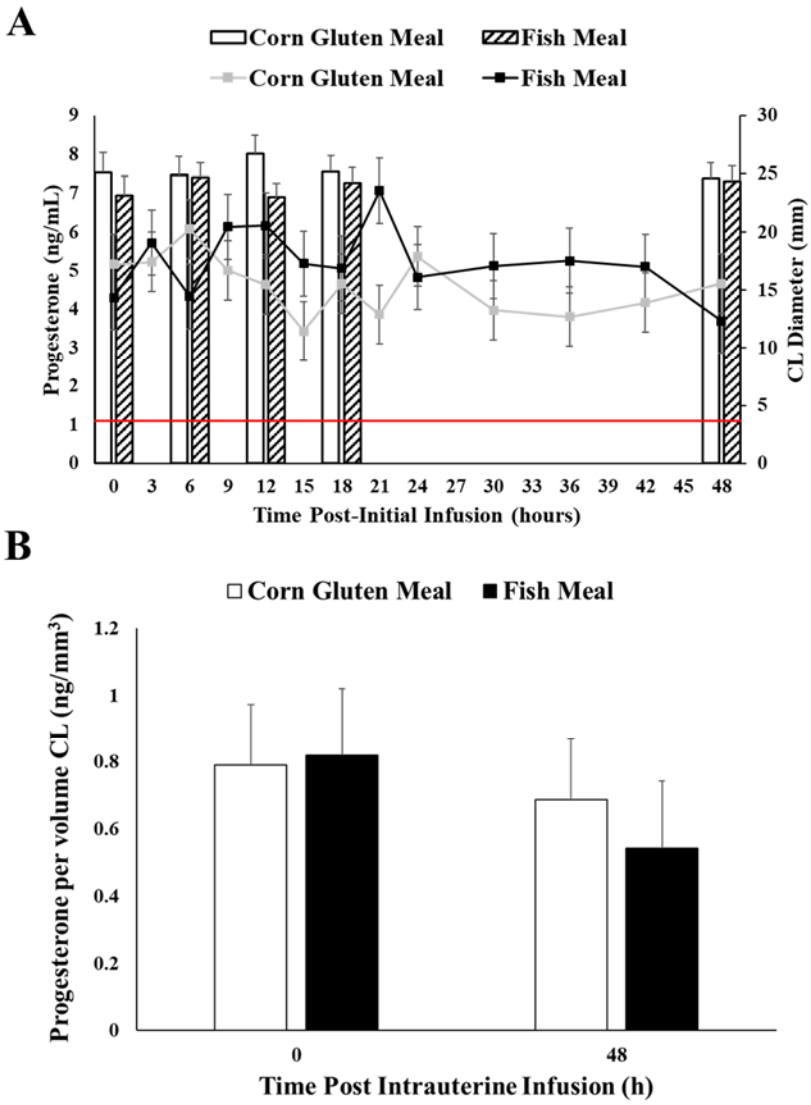

Figure 51. Effects of corn gluten meal or fish meal on serum progesterone and corpus luteum diameter following intrauterine infusions of saline. Panel A: Cows were administered four intrauterine infusions of 0.25 mL saline at 6-h intervals between day 10 to 12 following a synchronized estrus. Primary vertical axis represents progesterone concentration (ng/mL) of animals supplemented with corn gluten meal (grey line; n = 6) or fish meal (black line; n = 5) post-intrauterine infusions of saline. Red line represents progesterone concentration at 1ng/mL; progesterone concentrations below line are indicative of functional luteal regression. Dietary supplementation; $P > 0.10$; Time; $P > 0.10$; Dietary supplementation × Time; $P > 0.10$. Secondary vertical axis represented CL diameter (mm) of animals supplemented with corn gluten meal (open bar; n = 6) or fish meal (diagonal bar; n = 5) post-intrauterine infusions of saline. Dietary supplementation; $P > 0.10$; Time; $P > 0.10$; Dietary supplementation × Time; $P > 0.10$. Panel B: serum progesterone per CL volume (ng/mm^3) at 0 and 48 h post-intrauterine infusions of saline. Corn gluten meal (open bar; n = 6) or fish meal (solid bar; n = 5).

Cows administered 2 doses of $PGF_{2\alpha}$ at 12-h intervals resulted in two subpopulations based on serum progesterone – regressed and non-regressed CL. There was a stepwise decrease in serum progesterone for both cows having regressed and non-regressed CL from 0 to 21 h ($P < 0.05$). Serum progesterone continued to decrease between 18 to 48 h in cows with a regressed CL at the end of the experimental period, while there was a rebound in serum concentrations between 21 to 24 h for cows that had a functional CL. The gland was more sensitive to intrauterine infusion of $PGF_{2\alpha}$ for cows supplemented with corn gluten meal. Ninety percent of corn gluten meal supplemented animals had regressed CL within 48 h; yet, only 46% of the CL from cows supplemented with fish meal resulted in regression ($P < 0.05$; FIG 52A). One corn gluten supplemented animal infused with 2 doses of $PGF_{2\alpha}$ did not undergo functional regression; therefore, was removed from further statistical analysis.

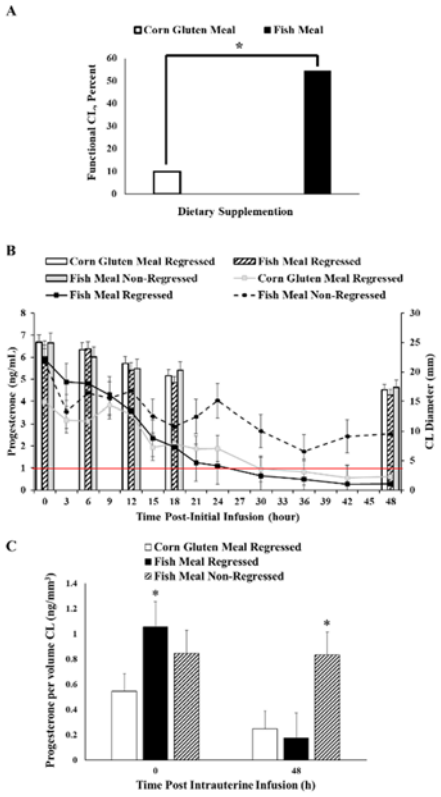

Figure 52. Effects of corn gluten meal or fish meal on serum progesterone and corpus luteum diameter following low-dose intrauterine infusions of prostaglandin F_{2alpha}. Cows were administered two intrauterine infusions of 0.5 mg prostaglandin (PG) $F_{2\alpha}$. between day 10 to 12 following a synchronized estrus. Cows were administered PGF_{2} at the first (0 h) and third (12 h) infusion and saline at the second (6 h) and forth infusion (18 h). Panel A: Percent of CL that were non-regressed following 2 intrauterine infusions of $PGF_{2\alpha}$. Non-regressed CL were defined as animals with serum progesterone > 1 ng/mL throughout experimental period. *Significance differences between dietary supplementation groups, $P < 0.05$. Panel B: Primary vertical axis represents progesterone concentration (ng/mL) of animals supplemented with corn gluten meal (regressed; grey line; n = 10) or fish meal (regressed; black line; n = 5; non-regressed; dash line; n = 6) post-intrauterine infusions of $PGF_{2\alpha}$. Red line represents progesterone concentration at 1ng/mL; concentrations below line were indicative of functional luteal regression. Dietary supplementation; $P > 0.10$; Time; $P < 0.05$; Dietary supplementation × Time; $P < 0.05$. Secondary vertical axis represented CL diameter (mm) of animals supplemented with corn gluten meal (regressed; open bar; n = 10) or fish meal (regressed; diagonal bar; n = 5; non-regressed; grey bar; n = 6) following intrauterine infusions of $PGF_{2\alpha}$. Dietary supplementation × time interaction, $P < 0.05$. Panel C: serum progesterone per CL volume (ng/mm^3) at 0 and 48 h post-intrauterine infusions of $PGF_{2\alpha}$. Corn gluten meal (regressed; open bar; n = 10) or fish meal (regressed; solid bar; n = 5; non-regressed; dashed bar; n = 6). *Significance differences between dietary supplementation subpopulations (corn gluten regression, fish meal regressed, or fish meal non-regressed) within time, $P < 0.05$.

Effects of Dietary Supplementation of Fish Meal on Luteal Diameter Following Intrauterine Infusions of $PGF_{2\alpha}$

Trans-rectal ultrasonography was performed to determine effects of dietary supplementation on luteal diameter following intrauterine infusions of $PGF_{2\alpha}$. Dietary supplementation did not affect initial CL diameter (0 h) regardless of treatment group ($P > 0.05$). Additionally, there were no effects of dietary supplementation on CL diameter throughout the 48-h sampling period for those animals infused with 4 doses of saline ($P > 0.05$; FIG 51). However, in corn gluten meal supplemented animals infused with 2 doses of $PGF_{2\alpha}$, diameter of the gland decreased by 12 h when compared to 0 h ($P < 0.05$; FIG 52B) and remained smaller for remainder of study. In fish meal supplemented animals infused with 2 doses of $PGF_{2\alpha}$, there was a decrease in gland diameter by 18 h for regressed ($P < 0.05$; FIG 52B) and 12 h for non-regressed CL ($P < 0.05$; FIG 52B), when compared to 0 h. Regardless of regression or non-regression, there was no difference in CL diameter at 48 h in fish meal supplemented animals receiving 2 doses of $PGF_{2\alpha}$ ($P > 0.05$), but were smaller when compared to 0 h ($P <0.05$).

Discussion

The present study was undertaken to examine the influence of dietary fish meal supplementation on luteal sensitivity to intrauterine infusions of $PGF_{2\alpha}$. Fish oil and meal are high in omega-3 fatty acids, which has been reported to positively influence reproductive success in the bovine (40, 134, 219). However, the central mechanism by which fish by-products exert beneficial effects on reproductive performance is still largely unknown. To our knowledge, this is the first study to examine the influence of fish meal supplementation on luteal sensitivity to $PGF_{2\alpha}$ in the bovine. Herein, we

provide for the first-time, evidence that fish meal supplementation decreases luteal sensitivity to uterine infusions of $PGF_{2\alpha}$ in non-lactating beef cows.

It is well documented that nutrition influences reproductive performance and CL function, especially energy (247, 248). Animals in the present study had similar gain in BW during the supplementation period. Gains in BW were similar to a previous study from our laboratory using this experimental model (153). However, cows receiving fish meal supplementation had an increased plasms EPA and DHA beginning on day 7 and 14, respectively, and remained higher as compared to corn gluten meal supplemented animals. These results are consistent with previous studies from our laboratory (69, 72, 153) and another study (249) where supplementation of fish by-products increase plasma EPA and DHA. Therefore, alterations in luteal sensitivity to $PGF_{2\alpha}$ were most likely due to changes in dietary supplementation (i.e. long-chain fatty acids) and not attributed to increased caloric intake.

In the present study, initial serum progesterone concentrations were unaffected by dietary supplementation. Dietary supplementation did not influence serum progesterone in animals administered 4 doses of saline. These results agree with other reports in the literature where fish meal (40, 43, 249) and oil (219) had minimal effects on progesterone secretion by the CL. Intrauterine infusion of 2 doses of $PGF_{2\alpha}$ at 12-h intervals decreased serum progesterone from 0 to 15 h; and these changes in serum where similar to a previously reported study administering 2 doses of $PGF_{2\alpha}$ at 12-h (250). In the current study, 2 doses of $PGF_{2\alpha}$ at 12-h intervals resulted in functional regression for 90% of cows supplemented with corn gluten meal, while only 46% for animals supplemented with fish meal. There was a rebound in serum progesterone between 21 and 24 h for 54%

of cows supplemented with fish meal that had a functional CL at the end of the experimental period. These results demonstrate a protective action omega-3 fatty acids exert on the CL, altering luteal sensitivity to $PGF_{2\alpha}$ in a subpopulation of cows. However, these data do differ from a previously reported study using a similar experimental approach, wherein 50% luteal regression was observed (250). The differences between studies may be due to physiological status of animals. In the previous study, lactating dairy cows were utilized, while in the present study non-lactating beef cows were used. Lactation status has been shown to affect the hypothalamic–pituitary–gonadal axis in the bovine (251). Lactation has been shown to alter LH secretion (252, 253) and blood progesterone concentrations (254), thereby, possibly altering CL function and sensitivity to $PGF_{2\alpha}$ in lactating animals. Nonetheless, results from this study demonstrate supplementation of fish meal decreases luteal sensitivity to $PGF_{2\alpha}$ in a subpopulation of cows supporting our working hypothesis.

Dietary supplementation did not affect CL size at the start of the experimental period. These data are in agreement with another study that shows fish oil supplementation does not affect CL diameter (219). Additionally, size of CL did not change for cows receiving 4 doses of saline during the experimental period; while, diameter of the gland decreased in those cows administered 2 doses of $PGF_{2\alpha}$, regardless of dietary supplementation or functional status (regressed vs non-regressed) of the gland.

Generally, luteolysis is divided into two distinct physiological events: loss of progesterone synthesis (255) and structural regression of the gland (256, 257). In the current study, fish meal supplementation decreased luteal sensitivity in 54% of cows infused with $PGF_{2\alpha}$. Surprisingly, although serum progesterone was maintained above 1

ng/mL, indicative of a functional CL, diameter of the gland decreased. The decrease in CL size without functional regression is interesting. Prostaglandin $F_{2\alpha}$ is a potent vasoconstrictor (258, 259) and the decrease in CL size may due to a decrease in ovarian or luteal blood flow. However, this is probably not the cause as recent studies have been reported in the literature that show an increase in blood following $PGF_{2\alpha}$ administration (260, 261).

Another possible mechanism for decreased size of CL may be the effects of $PGF_{2\alpha}$ on immune cell number or activity. The CL is populated with immune cells (262-264) and specific type of immune cell varies with stage of cycle (263, 265). Regulatory T lymphocytes are present in mature CL and these cells may secrete cytokines such as IL-4 and -10, which may aid in the maintenance of the CL (266, 267). However, reduction in progesterone following a pulse of $PGF_{2\alpha}$ may decrease number of regulatory T lymphocytes and thereby allowing for infiltration of autoreactive immune cells that secrete interferon-γ and tumor necrosis factor alpha (268, 269). Both of these cytokines have been shown to play an active role in regression of the CL (270), which possibly activates direct and/or indirect pathways leading to structural regression of the gland, as reviewed by Pate and Keyes (271). While gland size decreased in both $PGF_{2\alpha}$ subpopulations in fish meal supplemented cows, it is unclear whether decrease of gland size was a result of structural regression, or indirect responses following infusion of $PGF_{2\alpha}$ (i.e. changes in immune cell numbers or type). Additional studies are required to determine, expression of apoptotic genes, as well as changes in immune cell populations and cytokine secretion following intrauterine infusion of $PGF_{2\alpha}$.

Dietary supplementation of fish meal has been shown to improve pregnancy rates in beef (198) and dairy cows (134, 135, 199, 200). Researchers have hypothesized that incorporation of omega-3 fatty acids into uterine tissues alter prostaglandin secretion (43, 88, 272), thereby improving reproduction in the bovine. Omega-3 fatty acids not only influence uterine, but luteal function as well. In the present study, fish meal supplementation decreased the luteal sensitivity to $PGF_{2\alpha}$, which may contribute to improved fertility. The decrease in luteal sensitivity may be due to alternations of lipid microdomains and/or increased lateral mobility of the bovine FP receptor due to incorporation of omega-3 fatty acids into plasma membrane (153). Results from this study bridge the gap in knowledge on potential mechanisms by which omega-3 fatty acids increase reproductive success in beef and dairy cows.

CHAPTER 8

CONCLUSIONS

It is well-documented that a considerable number of pregnancies are lost during the period of maternal recognition of pregnancy. Many reproductive biologists have postulated that slow developing embryos may fail to establish maternal recognition of pregnancy because the signal is too weak or too late to effectively block uterine $PGF_{2\alpha}$ secretion. A single pulse of uterine $PGF_{2\alpha}$ may initiate the cellular and molecular pathways that lead to the regression of the CL and loss of the pregnancy. The *in vitro* and *in vivo* studies that we conducted and reported herein provide compelling evidence that incorporation of omega-3 polyunsaturated fatty acids from fish by-products into biological membranes influence luteal membrane dynamics and the FP receptor lateral mobility, $PGF_{2\alpha}$-induced intracellular signaling, receptor internalization, and decreases luteal sensitivity to $PGF_{2\alpha}$. The inclusion of fish by-products in the diet of breeding cattle may reduce luteal sensitivity to $PGF_{2\alpha}$ and increase the window of opportunity for slow developing embryos to trigger maternal recognition of pregnancy. Breeding trials are warranted to determine if dietary supplementation improves pregnancy rates in breeding cows.

In vitro experiments were also conducted to investigate individual omega-3 polyunsaturated fatty acids both EPA and DHA on luteal membrane function. Most studies reported in the literature show that DHA may have a greater influence on the

disruption of lipid microdomains (54, 87, 96, 150, 151), despite the fact that EPA alters microdomain structure and lipid composition as well (149). Surprisingly, both EPA and DHA exert equivalent effects on lipid microdomains nanostructure in a dose-dependent manner, Chapter 4, Figure 29. Alterations in lipid microdomain structure by individual omega-3 fatty acids also translated to increased lateral mobility and decreased resident times of FP receptors. There are sources of algae that contain high levels of DHA and future whole animal experiments should be conducted to investigate individual sources of omega-3 polyunsaturated fatty acids on luteal function.

It is well documented that supplementation of omega-3 fatty acids has beneficial effects on reproduction. However, the exact mechanism is unknown, especially with the time of maternal recognition of pregnancy. While the current studies show alterations in lipid microdomains and decreased luteal sensitivity to $PGF_{2\alpha}$, the effects fish meal supplementation exerts on CL progesterone biosynthesis and metabolism, embryonic interferon τ production, and attenuated $PGF_{2\alpha}$ signaling in response to a developing embryo within the 15-18 d window of embryonic development remain to be elucidated. A 'triangular interaction' (Figure 53) is proposed between embryonic interferon τ, endogenous uterine $PGF_{2\alpha}$, and the CL function in the breeding female bovine. Our results demonstrate the effects fish meal has on luteal sensitivity in response to intrauterine infusions of $PGF_{2\alpha}$. However, much work is still warranted to assess the effects of omega-3 fatty acid supplementation on 1) interferon τ concentrations, 2) interactions between embryonic interferon τ and the CL, as well as 3) embryonic interferon τ interaction between endogenous uterine $PGF_{2\alpha}$ secretion and CL function. The model illustrates interactions that warrant further investigation to fully understand

mechanistic effects of dietary supplementation of omega-3 polyunsaturated fatty acids on CL function. In addition to exploring the proposed interactions, much work is warranted to determine the effects of dietary supplementation of omega-3 polyunsaturated fatty acids on luteal differentiation and sensitivity to LH, lipid droplet storages and trafficking of cholesterol, steroidogenesis.

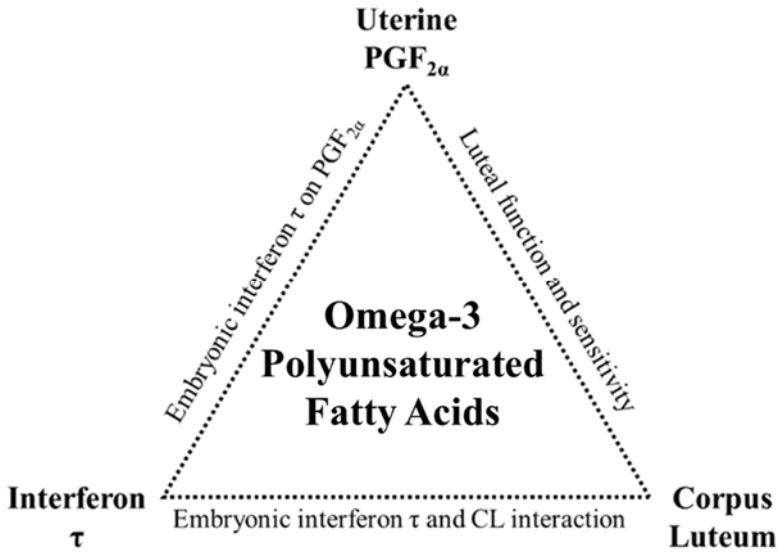

Figure 53. Interactions among uterine prostaglandin F_{2alpha}, corpus luteum, and embryonic interferon-tau during maternal recognition of pregnancy and influence of fish meal.

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APPENDIX A

INTITIUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

IACUC - IACUC Protocol ID: 16-8761AA (Bruemmer, Jason)
Protocol Title: Effect of omega-3 fatty acids on bovine luteal cell lipid microdomains and PGF2a Signaling

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COLORADO STATE UNIVERSITY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
ANIMAL USE APPLICATION

IACUC approval of this completed form is necessary prior to animals being obtained, housed or manipulated for research, testing or teaching purposes; performed at CSU or by CSU at other locations.

When you have completed all applicable sections of the protocol, you must also complete the certifications section and then click "Submit Form" link on the left-hand column.

All individuals listed on the protocol must have certified completion of the online CSU Animal Care and Use Training. Additionally, a "Training Record" should be uploaded in the Attachments section for the PI, Co-PI, and each person who will handle animals as a part of this study. Also, all individuals working with animals must be enrolled in the CSU Occupational Health and Safety Program (OHSP) via annual submission of a Risk Assessment Form to the OHSP.

Please contact an IACUC Coordinator if you have any questions.

Principal Investigator*

Name	Title	
Bruemmer, Jason	Professor	
Email	**EID**	**Phone**
Jason.Bruemmer@ColoState.EDU		(970) 491-3456
Department	**Mail Code**	
1171 Animal Sciences	1171	
Will PI work with animals as part of this project?		◉ Yes ○ No
Upload a "Training Record" for the PI under the "Attachments" section of this protocol.		

Co-Principal Investigator

Name	Title	
Engle, Terry	Professor	
Email	**EID**	**Phone**
Terry.Engle@ColoState.EDU		(970) 491-3597
Department	**Mail Code**	
1171 Animal Sciences	1171	
Will Co-PI work with animals as part of this project?		◉ Yes ○ No
Upload a "Training Record" for the Co-PI under the "Attachments" section of this protocol.		

Department Head

Name of Department Head	Degree	Title
Pond, Kevin		Professor
Email	**Phone**	**Fax**
Kevin.Pond@colostate.edu	(970) 491-7295	
Department Name	**Campus Delivery Code**	

Will the Department Head work with animals as a part of this project?		
If this person will work with animals as a part of this protocol, upload a "Training Record" for this individual under the "Attachments" section of this protocol.		○ Yes ◉ No

Administrative Contact

Name	Title	
Sellins, Karen		
Email	**EID**	**Phone**
Karen.Sellins@colostate.edu		(970) 689-2610
Department	**Mail Code**	
Will Administrative Contact work with animals as part of this project?		○ Yes ◉ No
If this person will work with animals as a part of this protocol, upload a "Training Record" for this individual under the "Attachments" section of this protocol.		

Other Submitter

Name	Title	
pdburns, pdburns	UNC	
Email	**EID**	**Phone**
Pat.d.Burns@colostate.edu		970 351 2695
Department	**Mail Code**	
Will this person be working with animals as a part of this project?		◉ Yes ○ No
If this person will work with animals as a part of this protocol, upload a "Training Record" for this individual under the "Attachments" section of this protocol.		

Other Personnel

	Name	Email	Title
☐	Graham, Peter	Peter.E.Graham@colostate.edu	
☐	Jessica Cidello	cedi7207@bears.unco.edu	
☐	Michele Plewes	micheleplewes@gmail.com	
☐	Hansen, Thomas	Thomas.Hansen@ColoState.EDU	
☐	Sinedino, Leticia	leticia.sinedino@colostate.edu	
☐	Mandujano, Julio	Julio.mandujano@unco.edu	

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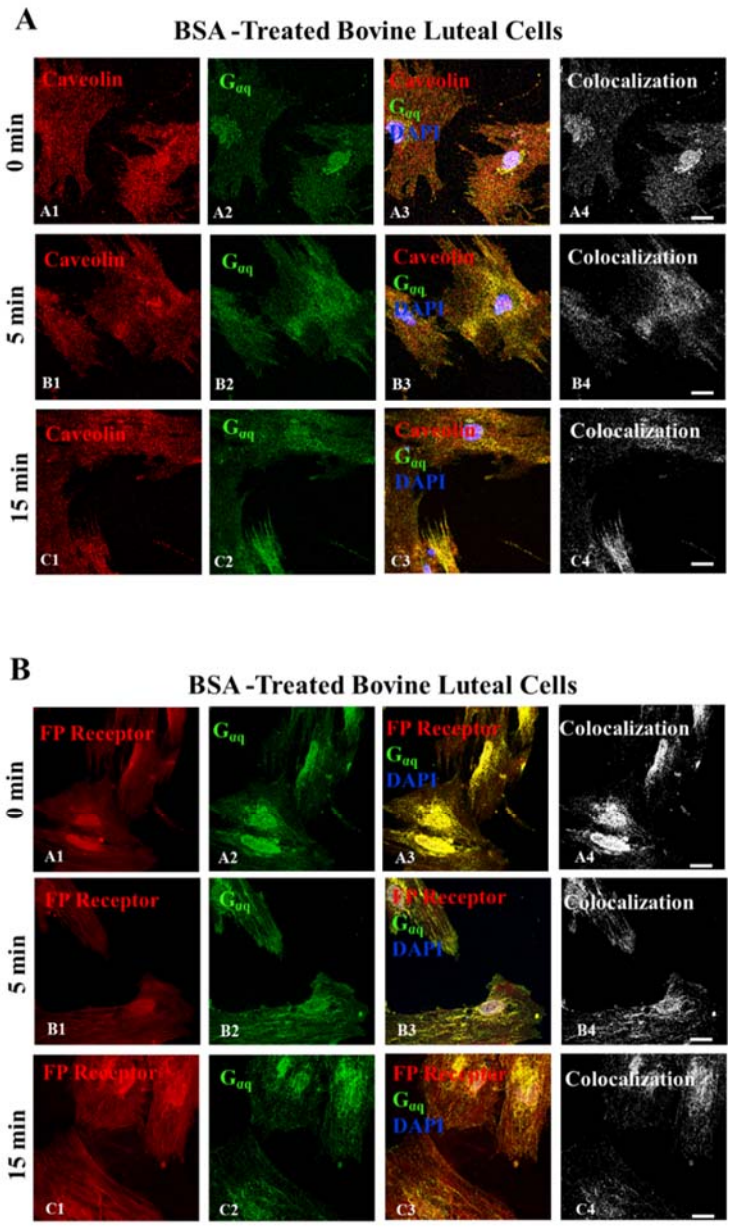
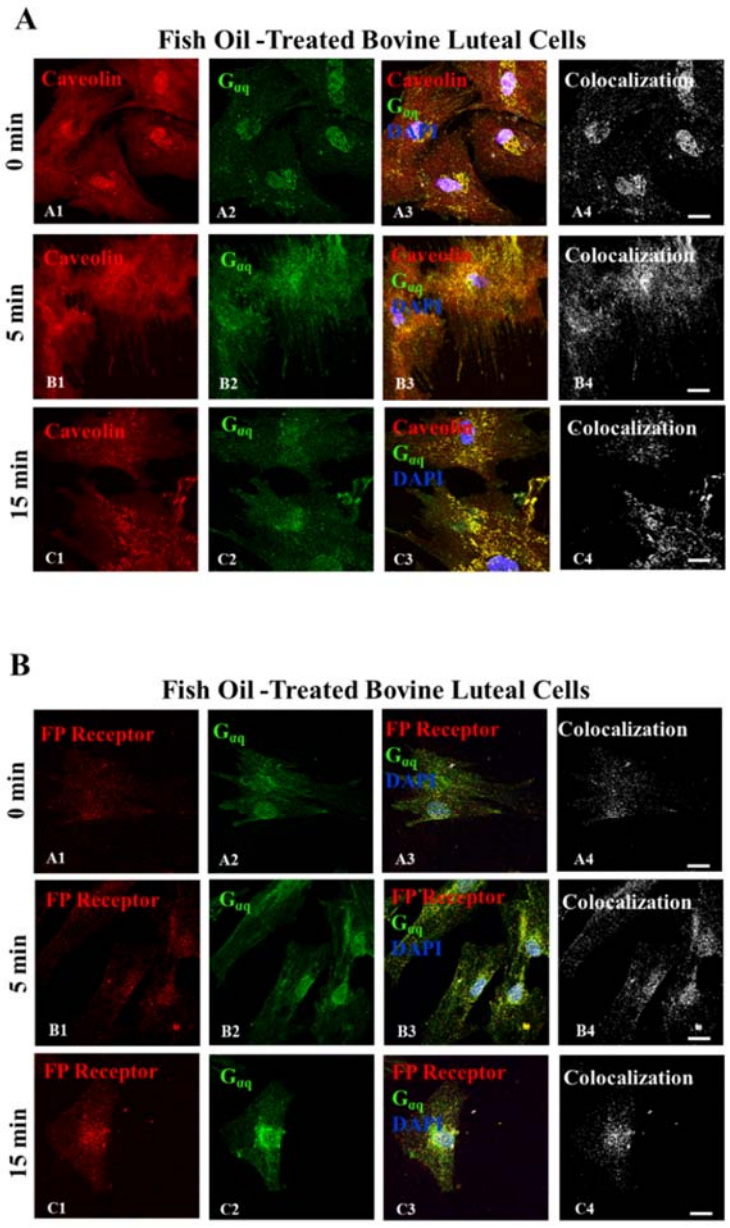
APPENDIX B

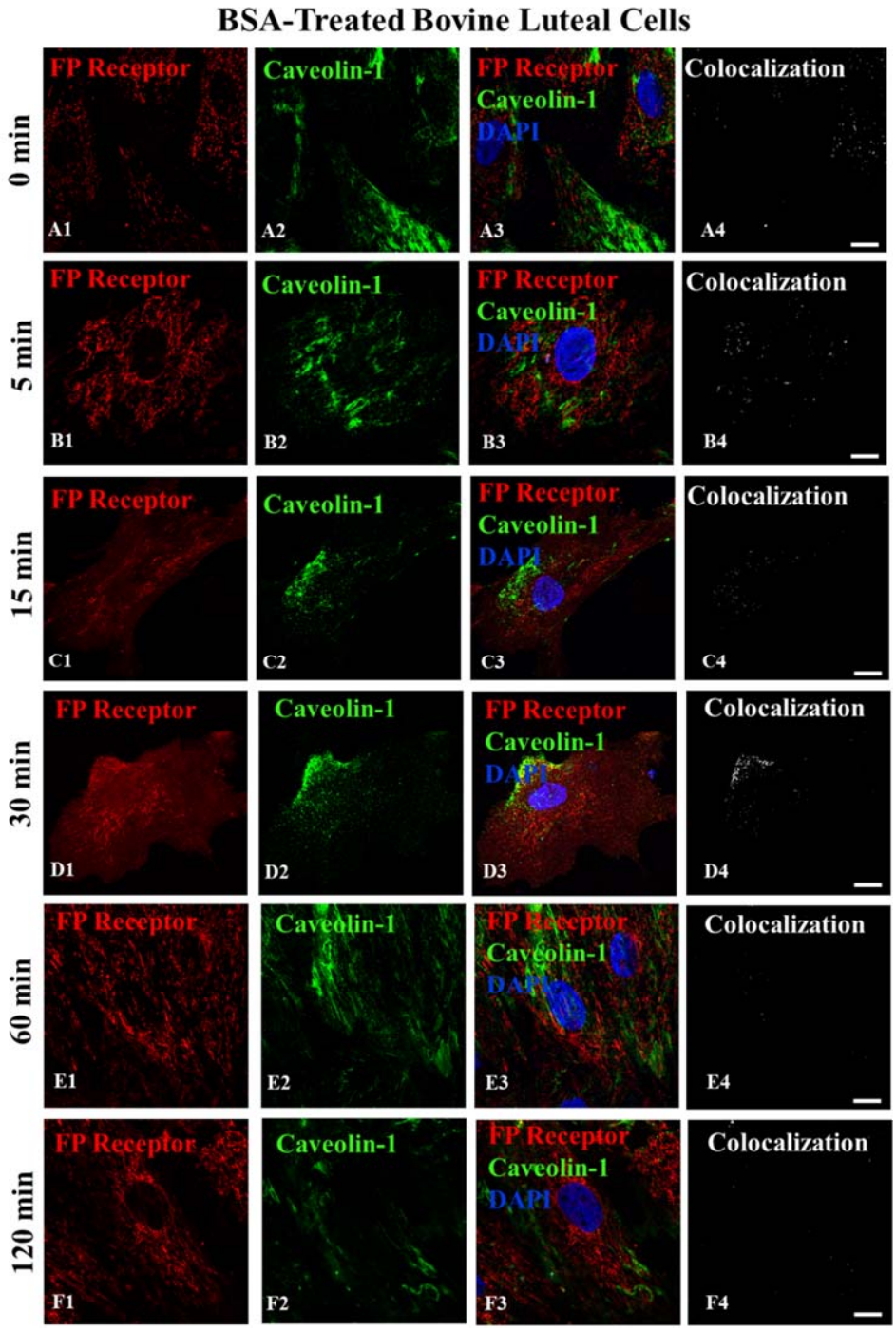
COLOCALIZATION OF G_{ALPHAQ} WITH CAVEOLIN-1 AND PROSTAGLANDIN F_{2ALPHA} RECEPTOR



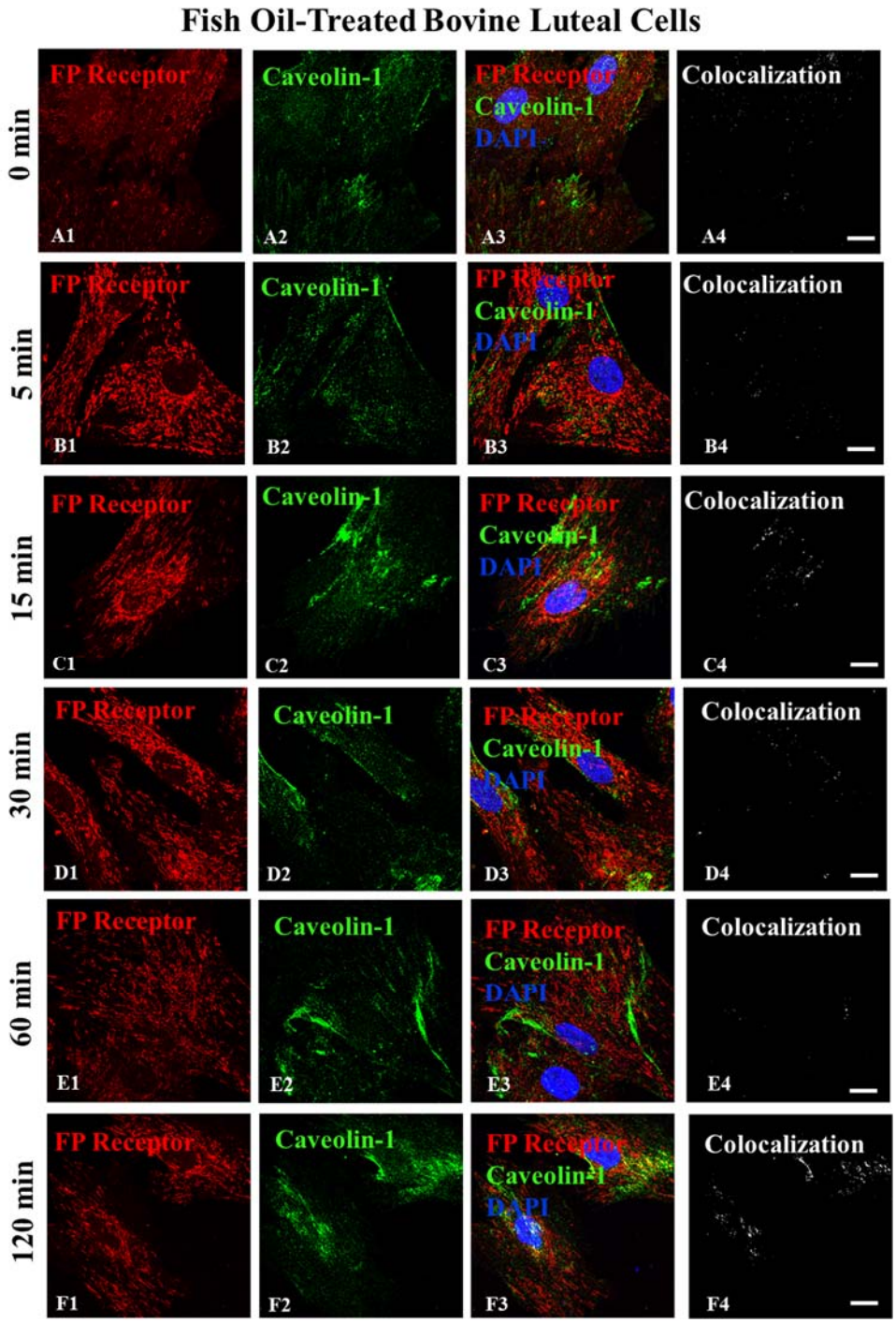
Appendix B1: Colocalization of $G_{\alpha q}$ with Caveolin-1 and FP receptor. Panel A shows representative micrographs of the colocalization of Caveolin-1 with $G_{\alpha q}$ obtained from cells treated with BSA following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, the time increases from 0 to 15 min as represented by individual letters (A-C; time indicated on left margin). From left to right numbers represent Caveolin-1 (1), $G_{\alpha q}$ (2), merge with Caveolin-1, $G_{\alpha q}$, and DAPI (3), colocalization (4). Panel B shows representative micrographs of the colocalization of FP receptor with $G_{\alpha q}$ obtained from cells treated with BSA following treatment with $PGF_{2\alpha}$. From top to bottom, time increases from 0 to 15 min as represented by individual letters (A-C; time indicated on left margin). From left to right, numbers represent FP receptor (1), $G_{\alpha q}$ (2), merge with FP receptor, $G_{\alpha q}$, and DAPI (3), colocalization (4). Micron bar represents 20 μm.



Appendix B2: Effects of fish oil on colocalization of $G_{\alpha q}$ with Caveolin-1 and FP receptor. Panel A shows representative micrographs of the colocalization of Caveolin-1 with $G_{\alpha q}$ obtained from cells treated with 0.03% (vol/vol) fish oil following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 15 min as represented by individual letters (A-C; time indicated on left margin). From left to right, numbers represent Caveolin-1 (1), $G_{\alpha q}$ (2), merge with Caveolin-1, $G_{\alpha q}$, and DAPI (3), colocalization (4). Panel B shows representative micrographs of the colocalization of FP receptor with $G_{\alpha q}$ obtained from cells treated with fish oil following treatment with $PGF_{2\alpha}$. From top to bottom, time increases from 0 to 15 min as represented by individual letters (A-C; time indicated on left margin). From left to right, numbers represent FP receptor (1), $G_{\alpha q}$ (2), merge with FP receptor, $G_{\alpha q}$, and DAPI (3), colocalization (4). Micron bar represents 20 μm.



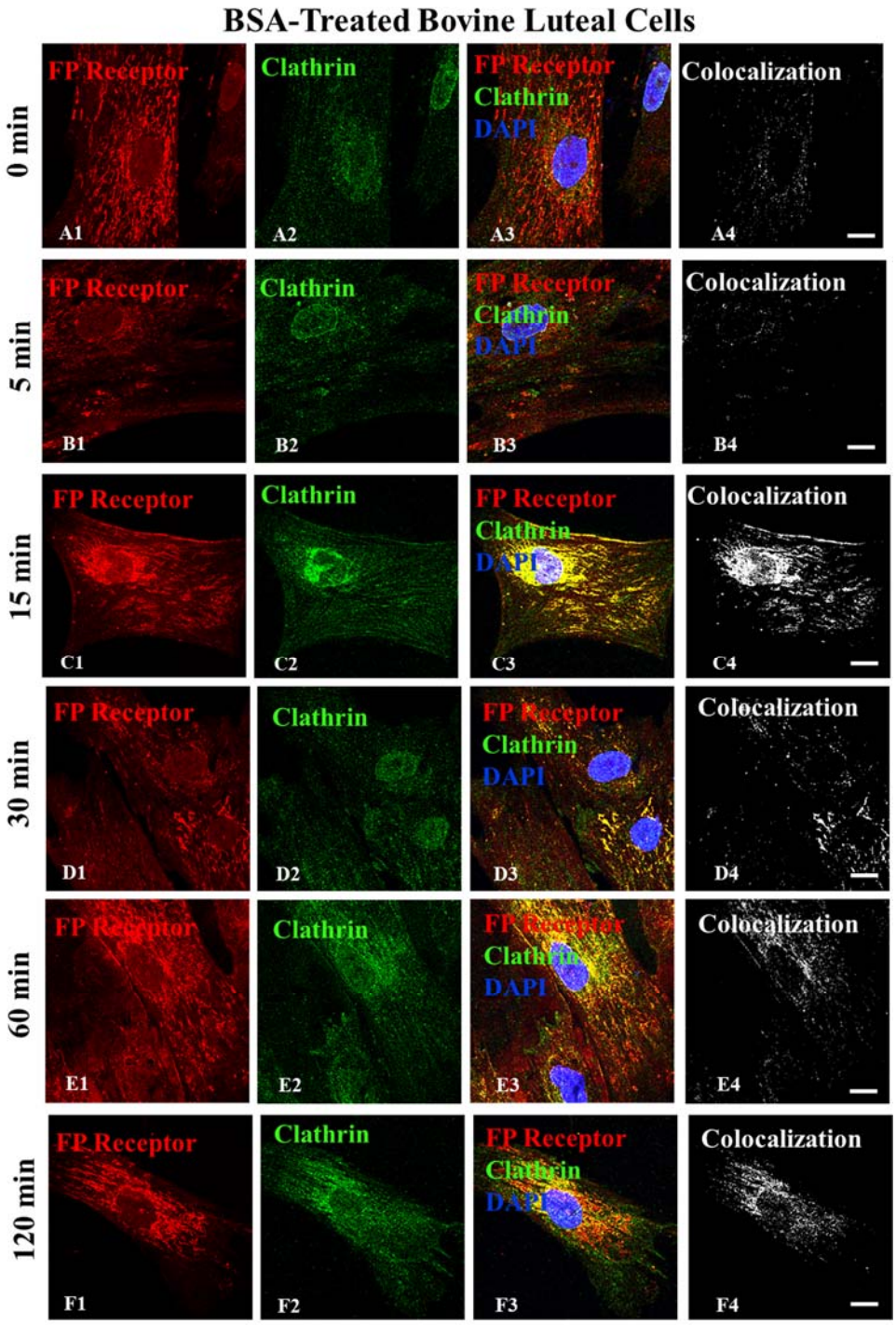
Appendix B3: Colocalization of FP receptor and Caveolin-1. Panel A shows representative micrographs of the colocalization of FP receptor with caveolin-1 obtained from cells treated with BSA following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), caveolin-1 (2), merge with FP receptor, caveolin-1, and DAPI (3), colocalization (4). Micron bar represents 20 μm.



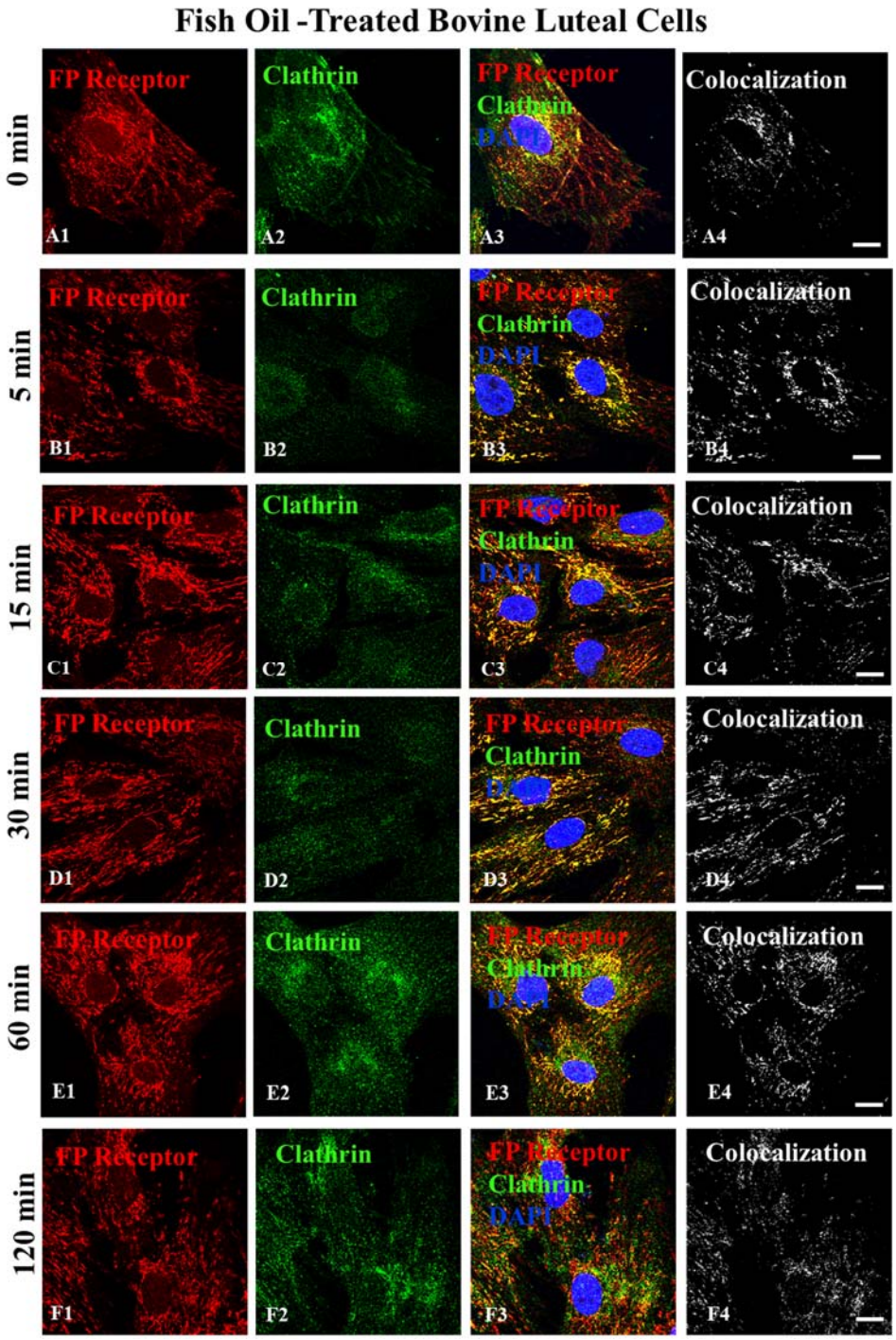
Appendix B4: Effects of fish oil on colocalization of FP receptor and Caveolin-1. Panel A shows representative micrographs of the colocalization of FP receptor with caveolin-1 obtained from cells treated with 0.03% (vol/vol) fish oil following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), caveolin-1 (2), merge with FP receptor, caveolin-1, and DAPI (3), colocalization (4). Micron bar represents 20 μm.

APPENDIX C

COLOCALIZATION CLATHRIN WITH THE PROSTAGLANDIN F_{2ALPHA} RECEPTOR



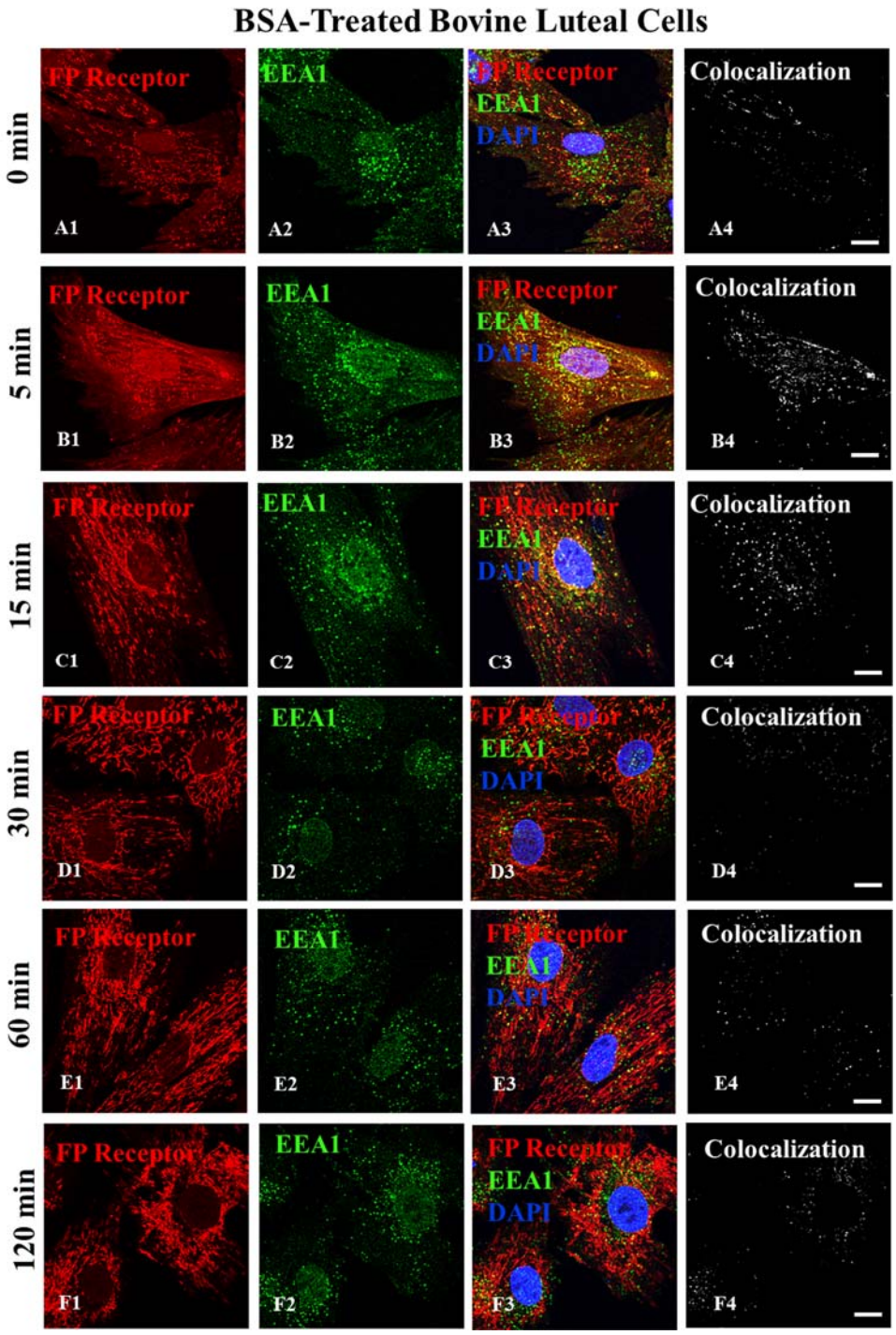
Appendix C1: Colocalization of FP receptor and Clathrin. Panel A shows representative micrographs of the colocalization of FP receptor with clathrin obtained from cells treated with BSA following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), clathrin (2), merge with FP receptor, clathrin, and DAPI (3), colocalization (4). Micron bar represents 20 μm.



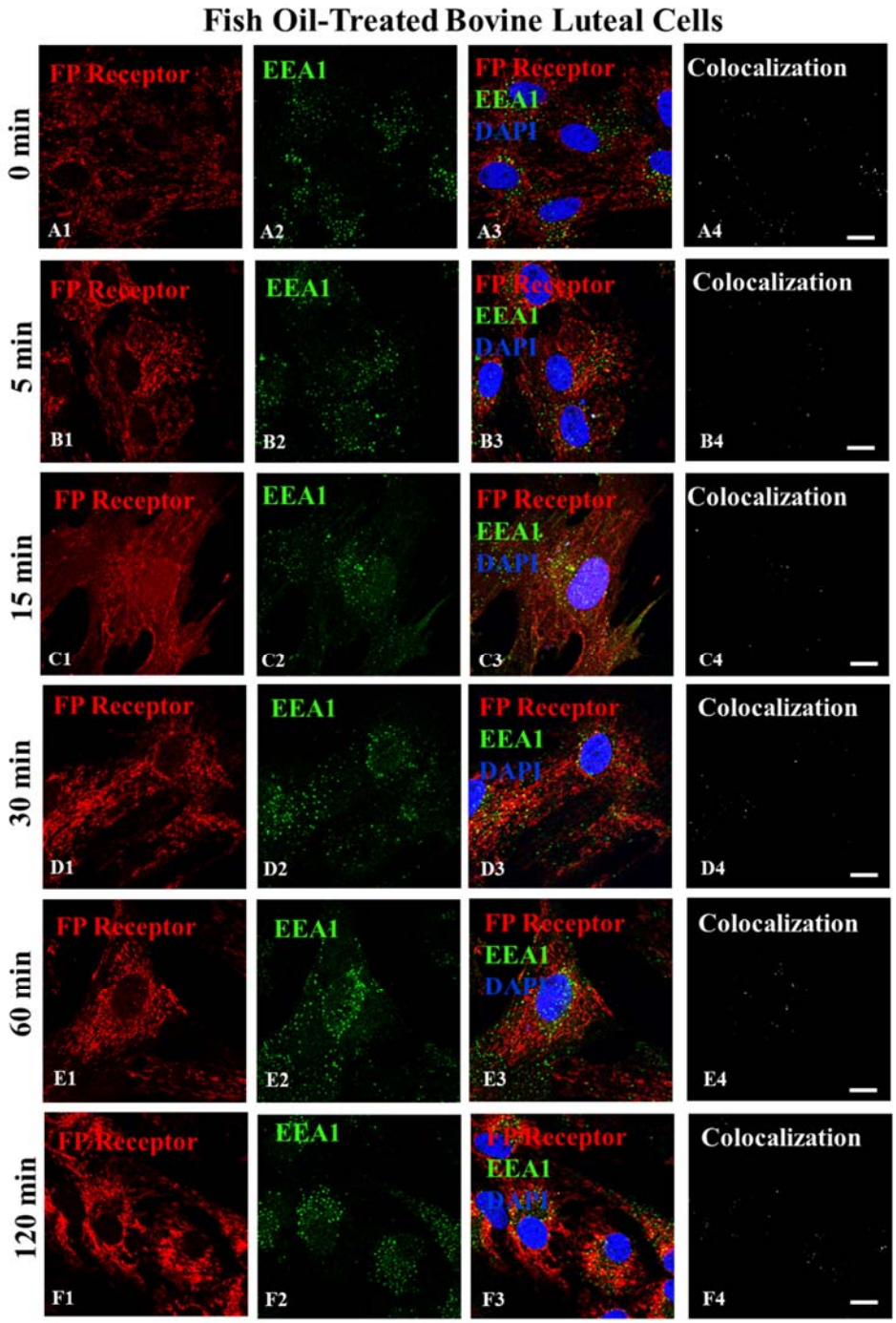
Appendix C2: Effects of fish oil on colocalization of FP receptor and Clathrin. Panel A shows representative micrographs of the colocalization of FP receptor with clathrin obtained from cells treated with 0.03% (vol/vol) fish oil following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), clathrin (2), merge with FP receptor, Clathrin, and DAPI (3), colocalization (4). Micron bar represents 20 μm.

APPENDIX D

COLOCALIZATION EARLY ENDOSOMAL ASSOCIATED PROTEIN WITH THE PROSTAGLANDIN F_{2ALPHA} RECEPTOR



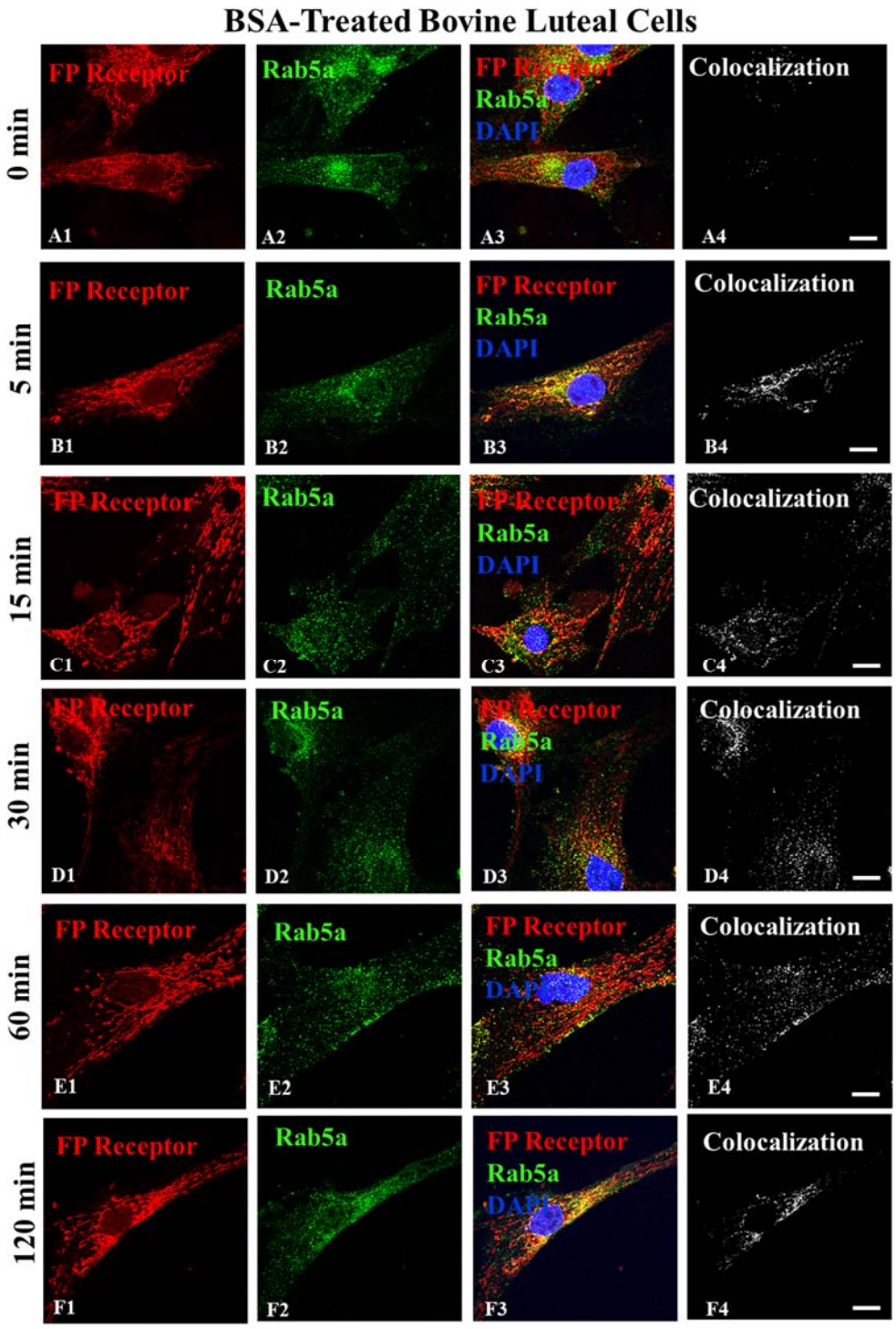
Appendix D1: Colocalization of FP receptor and EEA1. Panel A shows representative micrographs of the colocalization of FP receptor with EEA1 obtained from cells treated with BSA following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), EEA1 (2), merge with FP receptor, EEA1, and DAPI (3), colocalization (4). Micron bar represents 20 μm.



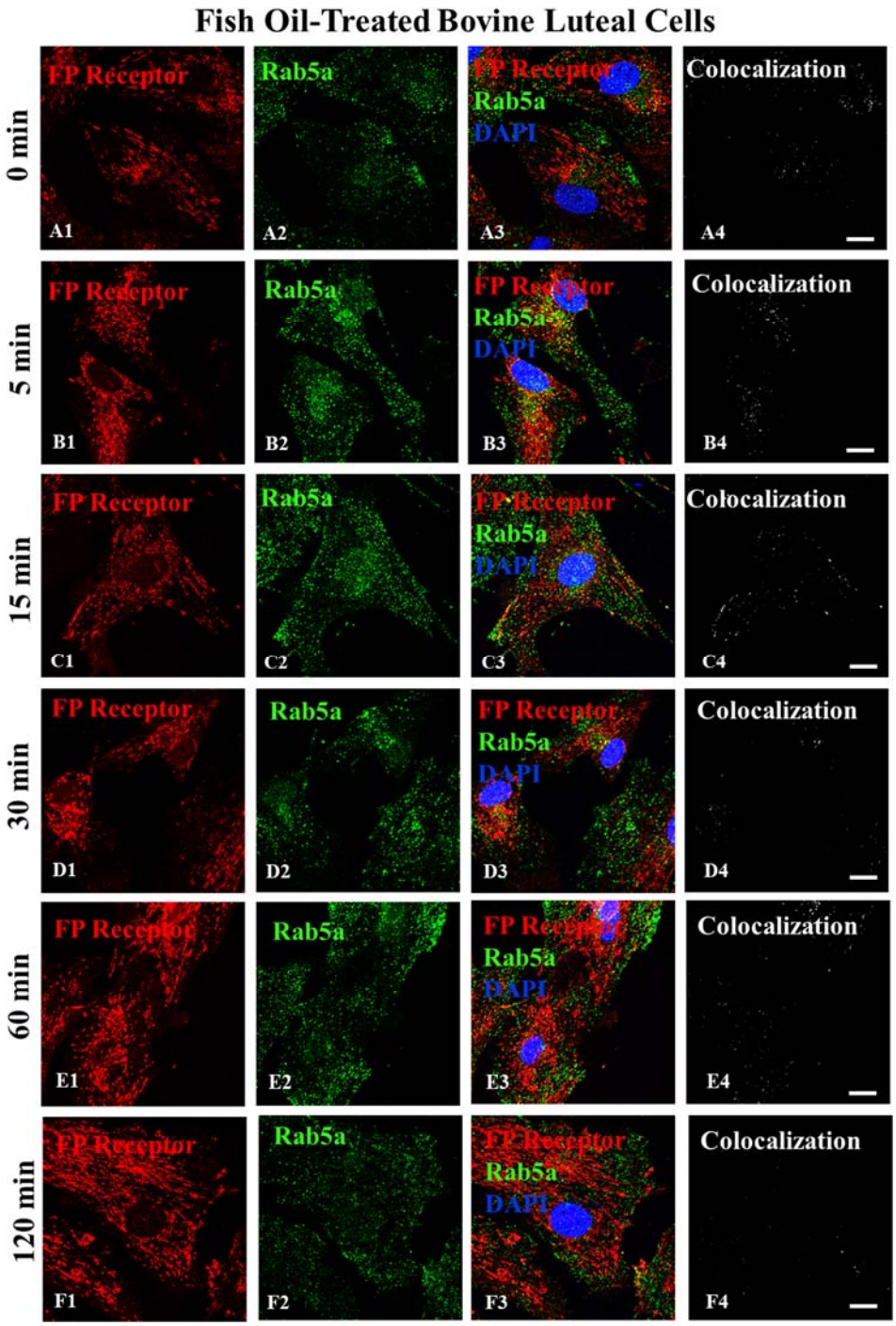
Appendix D2: Effects of fish oil on colocalization of FP receptor and EEA1. Panel A shows representative micrographs of the colocalization of FP receptor with EEA1 obtained from cells treated with 0.03% (vol/vol) fish oil following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), EEA1 (2), merge with FP receptor, EEA1, and DAPI (3), colocalization (4). Micron bar represents 20 μm.

APPENDIX E

COLOCALIZATION OF RAB5A WITH THE PROSTAGLANDIN F_{2ALPHA} RECEPTOR



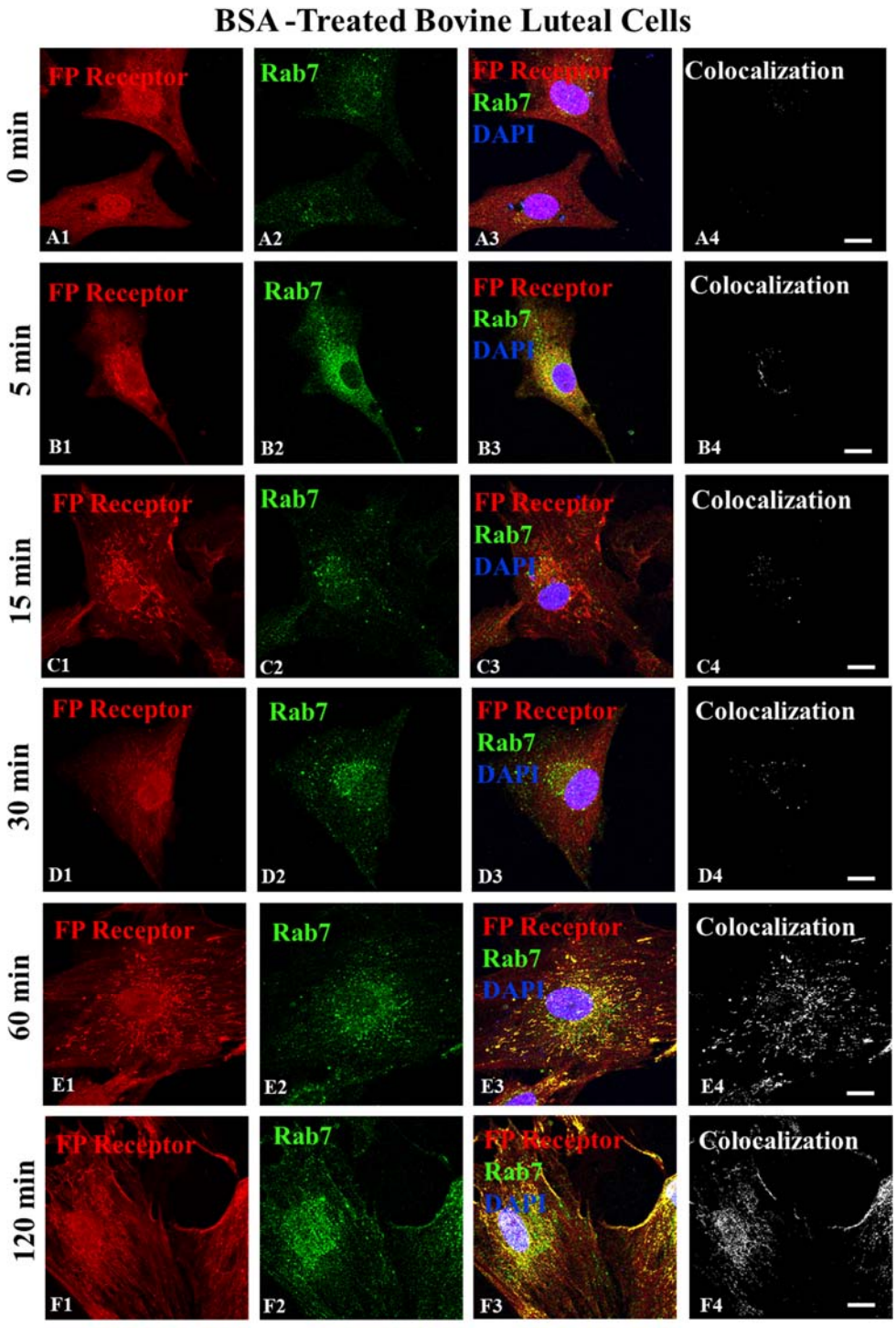
Appendix E1: Colocalization of FP receptor and Rab5. Panel A shows representative micrographs of the colocalization of FP receptor with Rab5 obtained from cells treated with BSA following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), Rab5 (2), merge with FP receptor, Rab5, and DAPI (3), colocalization (4). Micron bar represents 20 µm.



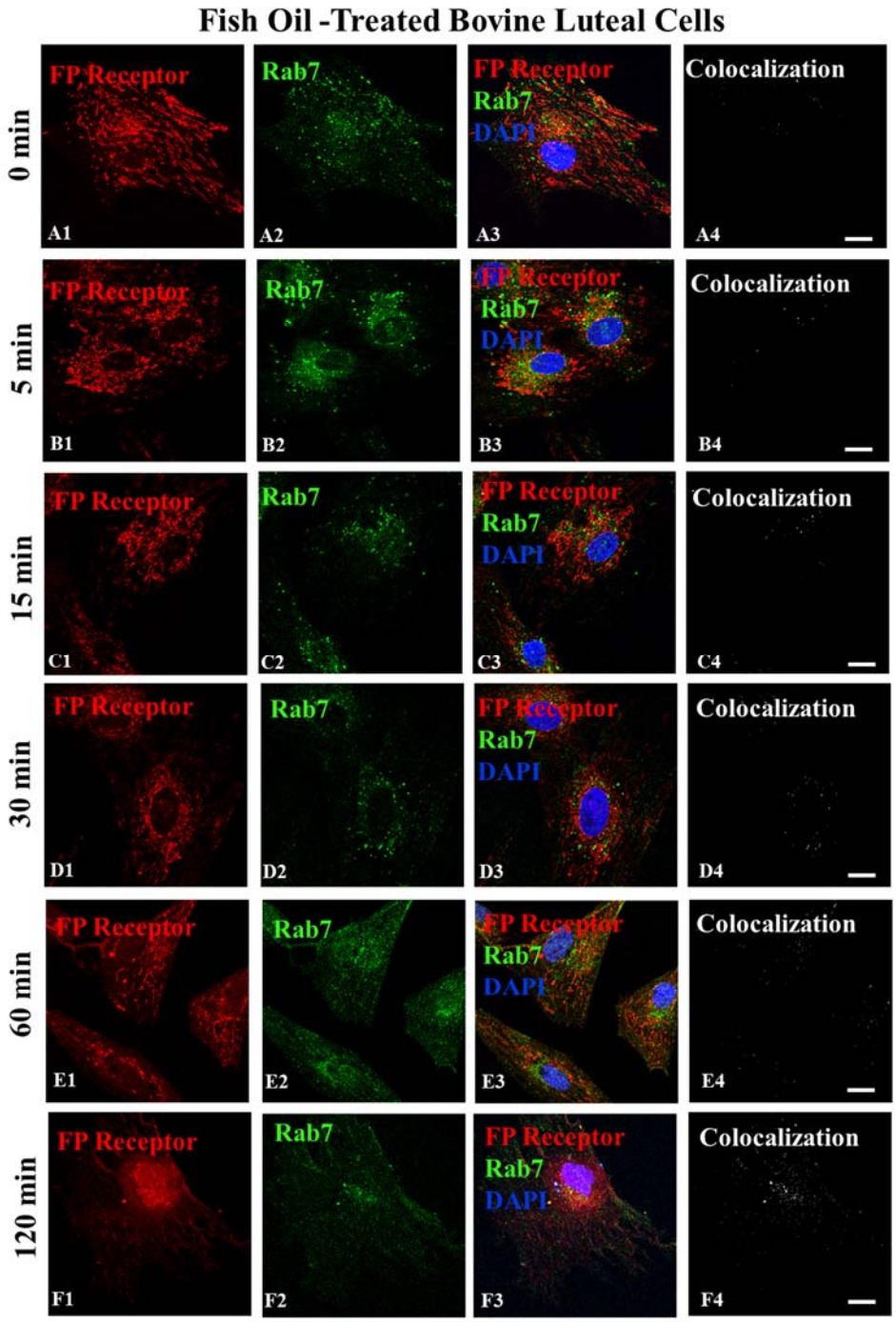
Appendix E2: Effects of fish oil on colocalization of FP receptor and Rab5. Panel A shows representative micrographs of the colocalization of FP receptor with Rab5 obtained from cells treated with 0.03% (vol/vol) fish oil following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), Rab5 (2), merge with FP receptor, Rab5, and DAPI (3), colocalization (4). Micron bar represents 20 μm.

APPENDIX F

COLOCALIZATION OF RAB7 WITH THE PROSTAGLANDIN F_{2ALPHA} RECEPTOR



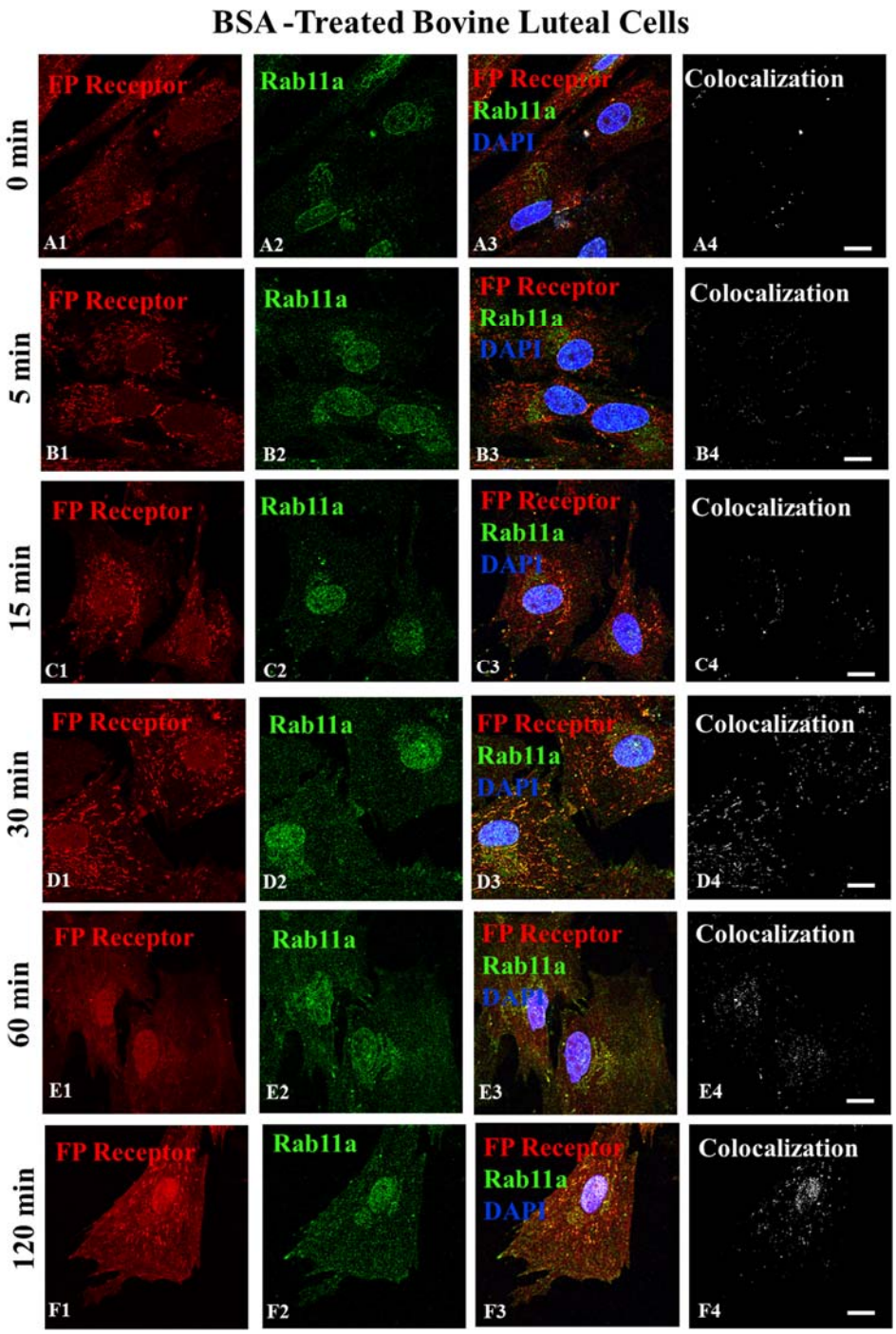
Appendix F1: Colocalization of FP receptor and Rab7. Panel A shows representative micrographs of the colocalization of FP receptor with Rab7 obtained from cells treated with BSA following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), Rab7 (2), merge with FP receptor, Rab7, and DAPI (3), colocalization (4). Micron bar represents 20 µm.



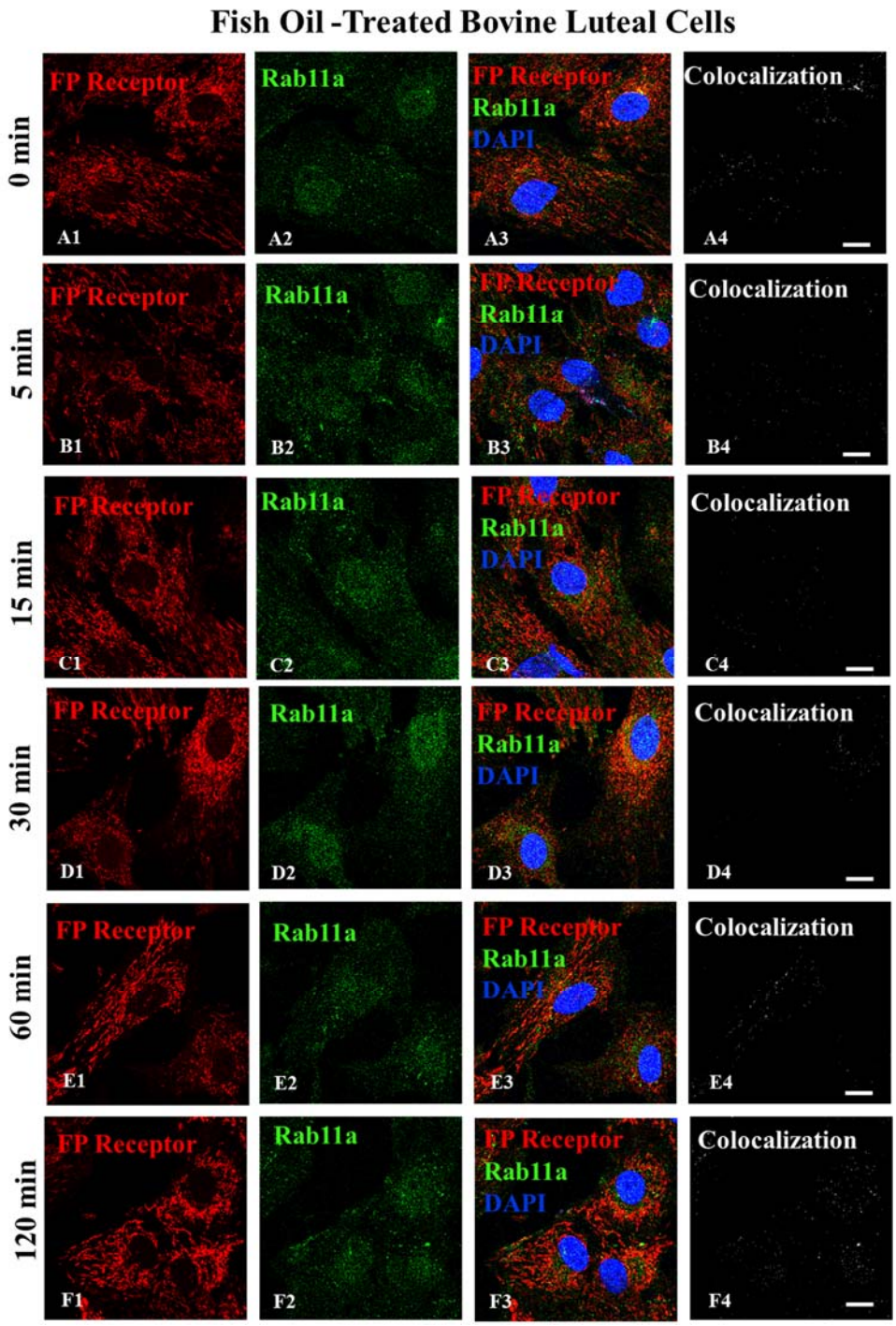
Appendix F2: Effects of fish oil on colocalization of FP receptor and Rab7. Panel A shows representative micrographs of the colocalization of FP receptor with Rab7 obtained from cells treated with 0.03% (vol/vol) fish oil following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), Rab7 (2), merge with FP receptor, Rab7, and DAPI (3), colocalization (4). Micron bar represents 20 µm.

APPENDIX G

COLOCALIZATION OF RAB11A1 WITH THE PROSTAGLANDIN F_{2ALPHA} RECEPTOR



Appendix G1: Colocalization of FP receptor and Rab11a. Panel A shows representative micrographs of the colocalization of FP receptor with Rab11a obtained from cells treated with BSA following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), Rab11a (2), merge with FP receptor, Rab11a, and DAPI (3), colocalization (4). Micron bar represents 20 μm.



Appendix G2: Effects of fish oil on colocalization of FP receptor and Rab11a. Panel A shows representative micrographs of the colocalization of FP receptor with Rab7 obtained from cells treated with 0.03% (vol/vol) fish oil following treatment with prostaglandin (PG) $F_{2\alpha}$. From top to bottom, time increases from 0 to 120 min as represented by individual letters (A-F; time indicated on left margin). From left to right, numbers represent FP receptor (1), Rab11a (2), merge with FP receptor, Rab11a, and DAPI (3), colocalization (4). Micron bar represents 20 μm.